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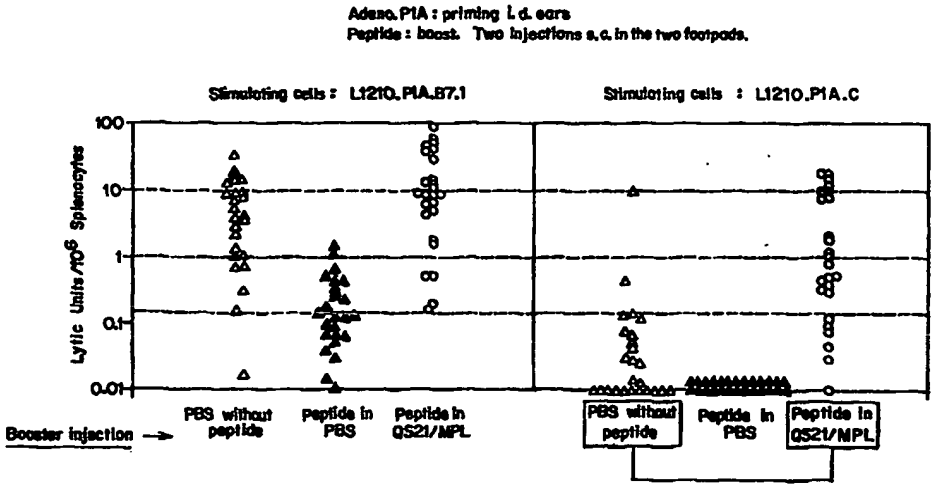

(57) Abstract

This invention relates to improved methods for modulating an immune response against an antigen using adenoviruses which express the antigen for priming immunization and antigen peptides for booster immunizations. Preferably the peptides are combined with QS21/MPL adjuvant. In particular, immunization methods for tumor antigens are provided. Kits for immunization are also provided.

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IMPROVED METHODS FOR INDUCING AN IMMUNE RESPONSE

Field of the Invention

This invention relates to improved methods for inducing immune responses against antigens using a virus vector for priming immunization and antigen peptides combined with an adjuvant for booster immunization. Methods for reducing an immune response using a virus vector for priming immunization and antigen peptides without an adjuvant for booster immunization are also provided.

Background of the Invention

The immune system of a mammal often provides the first line of defense against pathogenic organisms, as well as against tumors. The immune system recognizes antigens expressed by tumor cells or pathogens as foreign, i.e., "non-self". Upon recognition of a non-self antigen, an immune response is mounted against the antigen, resulting in antibodies and/or cytolytic T cells which recognize the antigen. The immune response of a mammal is also responsible to allergy (to antigens known as allergens) and autoimmune disease, which results from inappropriate recognition of host proteins as non-self.

Genes which are expressed in tumor cells, but not in normal counterparts, can be termed "tumor associated" genes. Typically, the host recognizes as foreign the products of tumor associated genes which are not expressed in normal non-tumorigenic cells. Thus, the expression of tumor associated genes can provoke an immune response against the tumor cells by the host. Tumor associated genes can also be expressed in normal cells within certain tissues without provoking an immune response. In such tissues, expression of the gene and/or presentation of an ordinarily immunologically recognizable fragment of the protein product on the cell surface may not provoke an immune response because the immune system does not "see" the cells inside these immunologically privileged tissues. Examples of immunologically privileged tissues include brain and testis.

The discovery of tumor associated genes and encoded proteins and peptides which are specifically recognized by the host immune system provides a source of immunogens for provoking an immune response against the tumor cell. The polypeptide products of tumor associated genes can be the targets for host immune surveillance and provoke selection and expansion of one or more clones of cytotoxic T lymphocytes (CTLs). Other tumor antigens are

encoded by differentiation antigens, introns of genes expressed in tumor cells and genes bearing point mutations in tumor cells. Examples of this phenomenon include proteins and fragments thereof encoded by the MAGE family of genes, the tyrosinase gene, the Melan-A gene, the BAGE family of genes, the GAGE family of genes, the RAGE family of genes, the MUM-1 gene, the CDK4 gene, and the brain glycogen phosphorylase gene. Thus, tumor associated expression of genes suggests that such genes can encode proteins which will be recognized by the immune system as foreign and thus provide a target for tumor rejection.

The process by which the mammalian immune system recognizes and reacts to foreign or alien materials is a complex one. An important facet of the system is the T cell response. This response requires that T cells recognize and interact with complexes of cell surface molecules, referred to as human leukocyte antigens ("HLA"), or major histocompatibility complexes ("MHCs"), and peptides. The peptides are derived from larger molecules which are processed by the cells which also present the HLA/MHC molecule. See in this regard Male et al., Advanced Immunology (J.P. Lipincott Company, 1987), especially chapters 6-10. The interaction of T cells and complexes of HLA/peptide is restricted, requiring a T cell specific for a particular combination of an HLA molecule and a peptide. If a specific T cell is not present, there is no T cell response even if its partner complex is present. Similarly, there is no response if the specific complex is absent, but the T cell is present. The mechanism is involved in the immune system's response to foreign materials, in autoimmune pathologies, and in responses to cellular abnormalities. Much work has focused on the mechanisms by which proteins are processed into the HLA binding peptides. See, in this regard, Barinaga, *Science* 257: 880, 1992; Fremont et al., *Science* 257: 919, 1992; Matsumura et al., *Science* 257: 927, 1992; Latron et al., *Science* 257: 964, 1992.

The mechanism by which T cells recognize cellular abnormalities has also been implicated in cancer. For example, in PCT application PCT/US92/04354, filed May 22, 1992, published on November 26, 1992, and incorporated by reference, a family of genes is disclosed, which are processed into peptides which, in turn, are expressed on cell surfaces, which can lead to lysis of the tumor cells by specific CTLs. The genes are said to code for "tumor rejection antigen precursors" or "TRAP" molecules, and the peptides derived therefrom are referred to as "tumor rejection antigens" or "TRAs". See Traversari et al., *J. Exp. Med.* 176:1453-1457, 1992; van der Bruggen et al., *Science* 254: 1643,1991; De Plaen et al., *Immunogenetics* 40:360-369, 1994 for further information on this family of genes. Also, see U.S. patent application serial

number 807,043, filed December 12, 1991, now U.S. Patent No. 5,342,774.

It has been demonstrated that cytotoxic T lymphocytes provide an effective response against tumor cells. Therefore, immunization which increases host cytotoxic T lymphocytes specific for one or more tumor rejection antigens can reduce tumor load in the host. The possible modes of delivery of tumor rejection antigens for immunization of cancer patients include: peptide with adjuvant (Feltkamp et al., *Eur. J. Immunol.* 23:2242-2249, 1993), protein with adjuvant, autologous dendritic cells or macrophages pulsed *in vitro* with the peptide or protein (Ossevoort et al. *J. Immunother.* 18:86-94, 1995), naked DNA or RNA (Conry et al., *Cancer Res.* 54:1164-1168, 1994), and recombinant defective viruses such as adenoviruses or poxviruses (Haddada et al, *Hum. Gene Ther.* 4:703-711, 1993; Irvine et al., *J. Immunol.* 154:4651-4657, 1995). Clinical trials of melanoma antigen-specific immunotherapy have thus far involved the use of either genetically modified tumor cells (Rosenberg et al., *J. Nat'l Cancer Inst.* 86:1159-1166, 1994; Cascinelli et al., *Human Gene Ther.* 5:1059-1064, 1994; Siegler et al., *Human Gene Ther.* 5:761-777, 1994) or the administration of synthetic antigenic peptides known to be presented in conjunction with defined MHC class I molecules (Jager et al., *Int. J. Cancer* 66:162, 1996; Marchand et al., *Int. J. Cancer* 63:883, 1995). An alternative approach to administration of synthetic peptides or genetically modified cells is the use of recombinant viral vectors to deliver the target antigens of choice. In this respect, human adenoviruses have been shown to function effectively as gene transfer vectors in a wide range of cells and tissues.

Recombinant viruses such as adenoviruses have potential as immunization vectors because they efficiently deliver genes into infected host cells. The use of adenoviruses as vaccine vectors against viral diseases (Cordier et al., *Gene Ther.* 2:16-21, 1995) has demonstrated their ability to elicit both humoral and cellular immune responses against proteins of other viruses (Randrianarison-Jewtoukoff and Perricaudet, *Biologicals* 23: 145-157,1995). The safety and variety delivery of modes of adenoviruses is well established (Top et al, *J. Infect. Dis.* 124:148-154, 1971). Other viral vectors also have been demonstrated to be safe and effective for delivery of nucleic acids into a host.

A mouse model system has been developed for determining the effectiveness of immunization protocols in eliciting a CTL response, and the potential for application of such protocols for use in human cancer immunotherapy. Mastocytoma P815 is a methylcholanthrene-induced mouse tumor. It expresses several antigens recognized by CTLs (Boon et al, *J. Exp. Med.* 152:1184-1193, 1980). Gene P1A, which codes for antigen P815A, is expressed in several

mastocytoma tumor lines (Van den Eynde et al., *J. Exp. Med.* 173:1373-1384, 1991), but not in normal tissues with the exception of testis. Accordingly, the P815A antigen represents a good mouse model for determining the effectiveness of immunization protocols against human tumor rejection antigens.

Immunization of a subject generally entails a "priming" or "primary" immunization which represents the first exposure of the subject to the immunogen, followed by one or more "boost" or "booster" immunizations with the same immunogen to provoke a more powerful and long-lasting immune response. It is known that an immune response is often generated to immunogenic epitopes of the particular adenovirus used as a vaccine vector. This effect limits the effectiveness of adenoviruses as vaccine vectors in that the adenovirus generally may not be used for both priming and boosting immunizations. Thus, a difficulty with the use of adenoviruses as vaccine vectors is providing a boosting vaccination to provoke a robust and long-lasting immune response.

Therefore, in order that a host immune system will maintain immune surveillance against tumor cells throughout life, there is a need for methods of immunization which are not limited by the host immune response against viral vectors such as adenovirus (typically neutralizing antibodies directed against the viral capsid proteins) used to immunize a subject. Further, there is a need to providing effective methods for immunizing against tumor antigens to provoke a cytotoxic T lymphocyte response in a host. There is also a need to reduce inappropriate immune responses, such as found in allergy and autoimmune disease. Reduction of host immune responses is also desirable to reduce allograft transplant rejection by the host.

It is an object of the invention to provide methods and compositions effective in enhancing the immune response of a mammalian subject against tumor cells expressing tumor rejection antigens and against pathogens, in subjects having any type of HLA antigen-presenting molecules. It is also an object of the invention to provide methods and compositions effective in reducing the immune response of a mammalian subject in allergy, autoimmune disease and allograft rejection. It is a further object of the invention to provide kits which facilitate administration of primary and booster immunizations.

The invention is elaborated upon further in the disclosure which follows.

Summary of the Invention

The invention provides improved methods of immunization using virus vectors which

provoke immune responses to antigens. The invention includes the use of antigen peptides in adjuvant for booster immunizations. These booster immunizations provoke a more robust immune response in a greater proportion of subjects. The invention also includes the use of antigen peptides in a non-adjuvant carrier for booster immunizations effective in reducing an immune response against such antigens. Further, the invention provides compositions and pharmaceutical preparations, contained in kit form, which enable the induction of an immune response against tumor antigens.

According to one aspect of the invention, methods for inducing an immune response in a mammal against an antigen are provided. The methods comprise administering to the mammal a virus containing a nucleic acid molecule encoding the antigen or its precursor in an amount effective to generate a first immune response. The methods further include administering at least one booster dose comprising at least one peptide in an adjuvant, wherein the at least one peptide includes the antigen, in a combined amount effective to enhance the first immune response. In certain embodiments, the nucleic acid molecule encodes an antigen, or a precursor or variant of the antigen, selected from the group consisting of tumor antigens and antigens characteristic of a pathogen. Preferably, the tumor antigen is selected from the group consisting of MAGE-1 (SEQ ID NOs:3 and 4), MAGE-3 (SEQ ID NOs:5-7), BAGE (SEQ ID NO:8), GAGE (SEQ ID NO:9), RAGE (SEQ ID NO:10), GnT-V (SEQ ID NOs:11 and 12), MUM-1 (SEQ ID NO:13), Tyrosinase (SEQ ID NOs:19, 20, 22, 35 and 36), DAGE (SEQ ID NO:33) and MAGE-6 (SEQ ID NO:34). Most preferably, the tumor antigen is a MAGE-3 encoded antigen recognized by HLA-A1 and represented as SEQ ID NO:5.

The choice of adjuvants can be of importance in promoting a strong immune response. Thus, in other embodiments, the adjuvant used in this aspect of the invention is selected from the group consisting of saponin adjuvants (e.g. QS21), preferably combined with monophosphoryl lipid A (MPL) or a derivative thereof, adjuvants based on emulsions, alum, complete and incomplete Freund's adjuvants, and montanide. Preferably, the adjuvant is QS21/MPL.

The doses of virus and/or peptide can be administered by a variety of routes. In some embodiments, the virus or the at least one peptide is administered by injection. Preferably, the injection is intradermal or subcutaneous.

The invention provides for administration of booster doses of immunogen to the mammal to enhance the first immune response to the antigen. In some embodiments, at least two booster doses of the at least one peptide in an adjuvant are administered. Preferably, four booster doses

are administered.

In certain embodiments, the virus is selected from the group consisting of adenoviruses, adeno-associated viruses, poxviruses including vaccinia viruses and attenuated poxviruses such as NYVAC, Semliki Forest virus, Venezuelan equine encephalitis virus, retroviruses, Sindbis virus, and Ty virus-like particle. Preferably, the virus is an adenovirus.

According to another aspect of the invention, methods for reducing an immune response in a mammal against an antigen are provided. The methods comprise administering to the mammal a virus containing a nucleic acid molecule encoding the antigen or its precursor, in an amount effective to generate a first immune response. The methods further include administering at least one booster dose comprising at least one peptide in a non-adjuvant carrier, wherein the at least one peptide includes the antigen, in an amount effective to reduce the first immune response. In certain embodiments, the nucleic acid molecule encodes an antigen, or a precursor or variant of the antigen, selected from the group consisting of allergens, allograft antigens and autoimmune antigens.

The doses of virus and/or peptide can be administered by a variety of routes. In some embodiments, the virus or the at least one peptide is administered by injection. Preferably, the injection is intradermal or subcutaneous.

The invention provides for administration of doses of antigen peptide in a non-adjuvant carrier to the mammal to reduce the immune response to the antigen or allergen. In some embodiments, at least two booster doses of the at least one peptide in a non-adjuvant carrier are administered. Preferably, four booster doses in a non-adjuvant carrier are administered.

In certain embodiments, the virus is selected from the group consisting of adenoviruses, adeno-associated viruses, poxviruses including vaccinia viruses and attenuated poxviruses, Semliki Forest virus, Venezuelan equine encephalitis virus, retroviruses, Sindbis virus, and Ty virus-like particle. Preferably, the virus is an adenovirus.

According to another aspect of the invention, a kit is provided. The kit comprises a first container containing an adenovirus which includes a nucleic acid molecule encoding a tumor antigen and a second container containing at least one peptide including the tumor antigen, sufficient to boost an immune response of a mammal to the tumor antigen. In certain embodiments, the second container further contains an adjuvant. In other embodiments, the kit further comprises a third container containing an adjuvant, or further comprises instructions for administering the at least one peptide with an adjuvant. In any of the foregoing kits, the adjuvant

preferably is selected from the group consisting of saponin adjuvants, preferably combined with monophosphoryl lipid A or a derivative thereof, adjuvants based on emulsions, alum, complete and incomplete Freund's adjuvants, and montanide. More preferably, the adjuvant is QS21/MPL.

The kits of the invention can be formulated to be useful for the treatment of a range of tumors. Thus, in certain embodiments, the tumor antigen encoded by the adenovirus and in peptide form is selected from the group consisting of MAGE-1 (SEQ ID NOs:3 and 4), MAGE-3 (SEQ ID NOs:5-7), BAGE (SEQ ID NO:8), GAGE (SEQ ID NO:9), RAGE (SEQ ID NO:10), GnT-V (SEQ ID NOs:11 and 12), MUM-1 (SEQ ID NO:13), Tyrosinase (SEQ ID NOs:19, 20, 22, 35 and 36), DAGE (SEQ ID NO:33) and MAGE-6 (SEQ ID NO:34). Most preferably, the tumor antigen is a MAGE-3 encoded antigen recognized by HLA-A1 and represented as SEQ ID NO:5.

The invention in another aspect involves compositions comprising an adenovirus including a nucleic acid which encodes a non-adenovirus antigen and an adjuvant. The antigen and adjuvant are as described in the foregoing aspects of the invention.

According to yet another aspect of the invention, a composition comprising a peptide antigen is provided. The peptide comprises non-antigen amino acids which increase the solubility of the peptide antigen in a solvent selected from the group consisting of adjuvants and non-adjuvant aqueous solutions. Preferably, the peptide comprises at least one N-terminal glutamic acid residue, and more preferably comprises two N-terminal glutamic acid residues.

These and other objects of the invention will be described in further detail in connection with the detailed description of the invention.

Brief Description of the Figures

Fig. 1 depicts the results of the immunizations described in Example 1.

Fig. 2 depicts the results of the immunizations described in Example 2.

Figs. 3 and 4 depict the results of the immunizations described in Example 3.

Detailed Description of the Invention

Previous studies of an adenovirus-based immunization protocol for induction of an immune response against tumor antigens resulted in induction of CTLs reactive against the

antigen encoded by the adenovirus (Warnier et al., *Int. J. Cancer* 67:303-310, 1996). A single injection of 10^8 or 10^9 pfu of recombinant adenovirus resulted in a CTL response in nearly 100% of the inoculated mice. However, this high percentage depended on *in vitro* restimulation of splenocytes taken from the mice with irradiated cells which expressed the P1A gene and the gene encoding the costimulatory molecule B7.1. When splenocytes were mixed with irradiated cells which expressed only the P815A antigen, only 40% of the same mice displayed a CTL response. Furthermore, the level of lytic activity was reduced 10-fold. Re-immunization with the same adenovirus was not efficient due to the presence of neutralizing antibodies against the adenovirus capsid proteins. Other studies have also detailed the limitations of adenovirus-based immunization protocols.

It has now been discovered that these limitations can be overcome by immunization methods which combine a priming injection of an virus which expresses an antigen to induce a first immune response, followed by booster injections of peptides containing the antigen. Surprisingly, if peptide is administered in combination with adjuvant, the immune response of the host is increased relative to the first immune response, but if peptide is administered in combination with a non-adjuvant carrier, the immune response of the host is decreased relative to the first immune response. Therefore the invention disclosed herein provides the unexpected result that the choice of an adjuvant or a non-adjuvant carrier can alter the effect of a peptide antigen on the immune response generated in a host. Thus the invention provides methods for the use of viruses in delivering antigen precursors to subjects to provoke a first immune response and peptides in adjuvant for boosting the first immune response. Other methods for reducing the first immune response by administering peptides in a non-adjuvant carrier are also provided.

The methods of the invention involve the administration of viruses encoding one or more antigens to a subject by standard modes of immunization. The administration of the virus vector serves as the priming immunization which induces an initial (first) immune response (the primary immune response) against the tumor antigen. The subsequent booster immunizations with peptide antigens in combination with adjuvant increase the responsiveness of the subject's immune system to the antigen by inducing the secondary immune response, which is characterized by the increased production of cytotoxic T lymphocytes relative to the primary immune response. This is useful for increasing a subject's immune response for the treatment of tumors and pathogens.

Exemplary tumor associated peptide antigens that can be expressed to induce or enhance

an immune response are derived from tumor associated genes and encoded proteins including MAGE-1, MAGE-2, MAGE-3, MAGE-4, MAGE-5, MAGE-6, MAGE-7, MAGE-8, MAGE-9, MAGE-10, MAGE-11, GAGE-1, GAGE-2, GAGE-3, GAGE-4, GAGE-5, GAGE-6, BAGE-1, RAGE-1, LB33/MUM-1, DAGE, NAG, MAGE-Xp2, MAGE-Xp3, MAGE-Xp4, tyrosinase, brain glycogen phosphorylase, Melan-A and MAGE-C1. For example, antigenic peptides characteristic of tumors include those listed in Table 1 below.

Table 1: Exemplary Booster Peptides

Gene	MHC	Peptide	Position	SEQ ID NO:
MAGE-1	HLA-A1	EADPTGHSY	161-169	3
	HLA-Cw16	SAYGEPRKL	230-238	4
MAGE-3	HLA-A1	EVDPIGHLY	168-176	5
	HLA-A2	FLWGPRALV	271-279	6
	HLA-B44	MEVDPIGHLY	167-176	7
BAGE	HLA-Cw16	AARAVFLAL	2-10	8
GAGE-1,2	HLA-Cw16	YRPRPRRY	9-16	9
RAGE	HLA-B7	SPSSNRIRNT	11-20	10
GnT-V	HLA-A2	VLPDVFIRC(V)	2-10/11	11,12
MUM-1	HLA-B44	EEKLIVVLF	exon 2/intron	13
		EEKLSVVLF (wild type)		14
CDK4	HLA-A2	ACDPHSGHFV	23-32	15
		ARDPHSGHFV (wild type)		16
β-catenin	HLA-A24	SYLDSGIHF	29-37	17
		SYLDSGIHS (wild type)		18
Tyrosinase	HLA-A2	MLLAVLYCL	1-9	19
	HLA-A2	YMNGTMSQV	369-377	20
	HLA-A24	AFLPWHRLF	206-214	21
	HLA-B44	SEIWRDIDF	192-200	22
	HLA-DR4	QNILLSNAPLGPQFP	56-70	23
	HLA-DR4	DYSYLQDSDPDSFQD	448-462	24

		HLA-A2	YMDGTMSQV	369-377	35
		HLA-B44	YEIWRDIDF	192-200	36
	Melan-A^{MART-1}	HLA-A2	(E)AAGIGILTV	26/27-35	25,26
		HLA-A2	ILTVILGVL	32-40	27
	$gp100^{Pmel117}$	HLA-A2	KTWGQYWQV	154-162	28
		HLA-A2	IDTQVPFSV	209-217	29
		HLA-A2	YLEPGPVTA	280-288	30
		HLA-A2	LLDGTATLRL	457-466	31
		HLA-A2	VLYRYGSFSV	476-485	32
	DAGE	HLA-A24	LYVDSLFFL	301-309	33
	MAGE-6	HLA-Cw16	KISGGPRISYPL	292-303	34

The peptides of SEQ ID NOs: 33, 34 and 36 are presented in US application serial no. 08/724,774, PCT application publication no. WO96/10577 and US application serial no. 08/713,354, respectively. Other exemplary peptides include those listed in U.S. patent applications 08/672,351, 08/669,590, 08/487,135, 08/530,569 and 08/880,693. Other examples will be known to one of ordinary skill in the art (for example, see Coulie, *Stem Cells* 13:393-403, 1995), and can be used in the invention in a like manner as those disclosed herein.

Antigens that are characteristic of pathogens include antigens derived from viruses, bacteria, parasites or fungi. Examples of important pathogens include *Vibrio cholerae*, enterotoxigenic *Escherichia coli*, rotavirus, *Clostridium difficile*, *Shigella* species, *Salmonella typhi*, parainfluenza virus, influenza virus, *Streptococcus pneumoniae*, *Borella burgdorferi*, HIV, *Streptococcus mutans*, *Plasmodium falciparum*, *Staphylococcus aureus*, rabies virus and Epstein-Barr virus.

Viruses in general include but are not limited to those in the following families: picornaviridae; caliciviridae; togaviridae; flaviviridae; coronaviridae; rhabdoviridae; filoviridae; paramyxoviridae; orthomyxoviridae; bunyaviridae; arenaviridae; reoviridae; retroviridae; hepadnaviridae; parvoviridae; papovaviridae; adenoviridae; herpesviridae; and poxyviridae.

Bacteria in general include but are not limited to: *P. aeruginosa*; *E. coli*; *Klebsiella* sp.; *Serratia* sp.; *Pseudomonas* sp.; *P. cepacia*; *Acinetobacter* sp.; S. *epidermis*; *E. faecalis*; *S. pneumoniae*; *S. aureus*; *Haemophilus* sp.; *Neisseria* sp.; *N. meningitidis*; *Bacteroides* sp.;

Citrobacter sp.; *Branhamella* sp.; *Salmonella* sp.; *Shigella* sp.; *S. pyogenes*; *Proteus* sp.; *Clostridium* sp.; *Erysipelothrix* sp.; *Lesteria* sp.; *Pasteurella multocida*; *Streptobacillus* sp.; *Spirillum* sp.; *Fusospirocheta* sp.; *Treponema pallidum*; *Borrelia* sp.; *Actinomycetes*; *Mycoplasma* sp.; *Chlamydia* sp.; *Rickettsia* sp.; *Spirochaeta*; *Legionella* sp.; *Mycobacteria* sp.; *Ureaplasma* sp.; *Streptomyces* sp.; *Trichomoras* sp.; and *P. mirabilis*.

Parasites include but are not limited to: *Plasmodium falciparum*, *P. vivax*, *P. ovale*, *P. malaria*; *Toxoplasma gondii*; *Leishmania mexicana*, *L. tropica*, *L. major*, *L. aethiopica*, *L. donovani*; *Trypanosoma cruzi*, *T. brucei*; *Schistosoma mansoni*, *S. haematobium*, *S. japonium*; *Trichinella spiralis*; *Wuchereria bancrofti*; *Brugia malayi*; *Entamoeba histolytica*; *Enterobius vermiculoarus*; *Taenia solium*, *T. saginata*; *Trichomonas vaginatis*, *T. hominis*, *T. tenax*; *Giardia lamblia*; *Cryptosporidium parvum*; *Pneumocytis carinii*; *Babesia bovis*, *B. divergens*, *B. microti*; *Isospora belli*, *I. hominis*; *Dientamoeba fragilis*; *Onchocerca volvulus*; *Ascaris lumbricoides*; *Necator americanis*; *Ancylostoma duodenale*; *Strongyloides stercoralis*; *Capillaria philippinensis*; *Angiostrongylus cantonensis*; *Hymenolepis nana*; *Diphyllobothrium latum*; *Echinococcus granulosus*, *E. multilocularis*; *Paragonimus westermani*, *P. caliensis*; *Chlonorchis sinensis*; *Opisthorchis felineus*, *O. viverrini*; *Fasciola hepatica*; *Sarcoptes scabiei*; *Pediculus humanus*; *Phthirius pubis*; and *Dermatobia hominis*.

Fungi in general include but are not limited to: *Cryptococcus neoformans*; *Blastomyces dermatitidis*; *Ajellomyces dermatitidis*; *Histoplasma capsulatum*; *Coccidioides immitis*; *Candida* species, including *C. albicans*, *C. tropicalis*, *C. parapsilosis*, *C. guilliermondii* and *C. krusei*; *Aspergillus* species, including *A. fumigatus*, *A. flavus* and *A. niger*; *Rhizopus* species; *Rhizomucor* species; *Cunninghammella* species; *Apophysomyces* species, including *A. saksenaea*, *A. mucor* and *A. absidia*; *Sporothrix schenckii*; *Paracoccidioides brasiliensis*; *Pseudallescheria boydii*; *Torulopsis glabrata*; and *Dermatophytes* species.

Specific examples of antigens characteristic of a pathogen include: the influenza virus nucleoprotein (residues 218-226, Fu et al., *J. Virol.* 71:2715-2721, 1997), antigens from Sendai virus and lymphocytic choriomeningitis virus (An et al., *J. Virol.* 71:2292-2302, 1997), the E1 protein of hepatitis C virus (Bruna-Romero et al., *Hepatology* 25:470-477, 1997), the virus envelope glycoprotein gp160 of HIV (Achour et al., *J. Virol.* 70: 6741-6750, 1996), amino acids 252-260 or the circumsporozite protein of *Plasmodium berghei* (Allsopp et al., *Eur. J. Immunol.* 26:1951-1958, 1996), the infleunza A virus nucleoprotein (residues 366-374, Nomura et al., *J. Immunol. Methods* 193:4149, 1996), the listeriolysin O protein of *Listeria monocytogenes*

(residues 91-99, An et al., *Infect. Immmun.* 64:1685-1693, 1996), the E6 protein (residues 131-140, Gao et al., *J. Immunol.* 155:5519-5526, 1995) and E7 protein (residues 21-28 and 48-55, Bauer et al., *Scand. J. Immunol.* 42:317-323, 1995) of human papillomavirus type 16, the M2 protein of respiratory syncytial virus (residues 82-90 and 81-95, Hsu et al., *Immunology* 85:347-350, 1995), the herpes simplex virus type 1 ribonucleotide reductase (Salvucci, et al., *J. Virol.* 69:1122-1131, 1995) and the rotavirus VP7 protein (Franco et al., *J. Gen. Virol.* 74:2579-2586, 1993).

The skilled artisan will appreciate that a variety of methods of administration are useful in this invention including *ex vivo* and *in vivo* adminstration; these are detailed further below. Any modes of administration which effectively deliver the adenovirus and the peptide in adjuvant can be used according to the invention. For administration of the peptide in adjuvant or in a non-adjuvant carrier, preferred methods include intradermal and subcutaneous administration. Although these are preferred embodiments, the invention is not limited by the particular modes of administration disclosed herein. Standard references in the art (e.g., *Remington's Pharmaceutical Sciences*, 18th edition, 1990) provide modes of administration and formulations for delivery of immunogens with adjuvant or in a non-adjuvant carrier.

As part of the immunization protocols, the peptide antigens (and optionally the adenoviruses) preferably are administered mixed with one or more adjuvants to induce an immune response or to increase an immune response. An adjuvant is a substance incorporated into or administered simultaneously with antigen which potentiates the immmune response. Adjuvants may enhance the immunological response by providing a reservoir of antigen (extracellularly or within macrophages), activating macrophages and stimulating specific sets of lymphocytes. Adjuvants of many kinds are well known in the art. Specific examples of adjuvants include MPL (SmithKline Beecham), a congener obtained after purification and acid hydrolysis of *Salmonella minnesota* Re 595 lipopolysaccharide; saponins including QS21 (SmithKline Beecham); a pure QA-21 saponin purified from *Quillja saponaria* extract, QS-7, QS-17, QS-18, and QS-L1 (So et al., *Mol. Cells* 7:178-186, 1997); incomplete Freund's adjuvant; complete Freund's adjuvant; montanide; and various water-in-oil emulsions prepared from biodegradable oils such as squalene and/or tocopherol. Preferably, the peptides are administered mixed with a combination of QS21/MPL. The ratio of QS21 to MPL typically will be about 1:10 to 10:1, preferably about1:5 to 5:1 and more preferably about 1:1. Typically for human administration, QS21 and MPL will be present in a vaccine formulation in the rang of

about 1 μg to about 100 μg. Other adjuvants are known in the art and can be used in the invention (*see, e.g.* Goding, *Monoclonal Antibodies: Principles and Practice*, 2nd Ed., 1986). Methods for the preparation of mixtures or emulsions of peptide and adjuvant are well known to those of skill in the art of vaccination.

Other agents which stimulate the immune response of the subject can also be administered to the subject. For example, cytokines are also useful in vaccination protocols as a result of their lymphocyte regulatory properties. Many cytokines useful for such purposes will be known to one of ordinary skill in the art, including interleukin-12 (IL-12) which has been shown to enhance the protective effects of vaccines (*Science* 268: 1432-1434, 1995), IL-18 and GM-CSF.

Administration of peptide antigens in a non-adjuvant carrier results in a reduced immune response relative to the immune response generated by administration of a virus encoding the antigen. Thus administration of peptide antigens in a non-adjuvant carrier such as phosphate buffered saline can be used to reduce a subject's immune response to such antigens. This is useful in the treatment of allergic responses and autoimmune diseases, as well as in treating allograft rejection. A non-adjuvant carrier is a carrier which does not potentiate the immune response of the antigen with which it is co-administered. Non-adjuvant carriers include phosphate buffered saline, normal saline, Ringer's solution, and other solutions which are not known to potentiate an immune response.

Antigens that are allergens are generally proteins or glycoproteins, although allergens may also be low molecular weight allergenic haptens that induce allergy after covalently combining with a protein carrier (Remington's Pharmaceutical Sciences). Allergens include antigens derived from pollens, dust, molds, spores, dander, insects and foods. Specific examples include: the major horse allergen Equ c1 (Gregoire et al., *J. Biol. Chem.* 271:32951-32959, 1996), the Hor v 9 pollen allergen from barley (Astwood et al., *Gene* 182:53-62, 1996), the major allergen of the domestic cat, Fel d 1 (Counsell et al., *J. Allergy Clin. Immunol.* 98:884-894, 1996), a major latex allergen, Hev b 5 (Salter et al., *J. Biol. Chem.* 271:25394-25399, 1996), a major allergen of salmon, Sal s 1 (Lindstrom et al., *Scand. J. Immunol.* 44:335-344, 1996), allergens of the house dust mite, *B. tropicalis* (Carabello et al., *J. Allergy Clin. Immunol.* 98:573-579, 1996; Der p 2, Chua et al., *Clin. Exp. Allergy* 26:829-837, 1996; Der p 2, Harris et al., *Int. Immunol.* 9:273-280, 1997), major allergens of velvet grass, Hol l 1 and Hol l 5 (Schramm et al., *Int. Arch. Allergy immunol.* 110:354-363, 1996), a major allergen of ryegrass, Lol p 9 (Blaher et

al., *J. Allergy Clin. Immunol.* 98:124-132, 1996), the predominant allergen of bovine dander, BDA20 (Mantyjarvi et al., *J. Allergy Clin. Immunol.* 97:1297-1303, 1996), a major allergen of Kentucky bluegrass pollen, rKBG60 (Zhang et al., *Immunology*, 87:283-290, 1996), and the hornet venom allergen Dol m 5 (King et al., *J. Allergy Clin. Immunol.* 99:630-639, 1997).

Examples of antigens characteristic of autoimmune disease include: antigens from human myelin basic protein (residues 110-118), proteolipid protein (residues 80-88), myelin-associated protein (residues 287-295, 509-517, 556-564) in multiple sclerosis (Tsuchida et al. *Proc. Natl. Acad. Sci USA* 91:10859-10863, 1994), the islet cell antigen ICA69 in diabetes (Karges et al., *Biochim. Biophys. Acta* 1360:97-101, 1997), the protein L7 in rheumatic diseases such as systemic lupus erythematosus , rheumatoid arthritis, and systemic sclerosis (Neu et al., *Arthritis Rheum.* 40:661-671, 1997), two peptides from human acetylcholine receptor alpha-subunit, p195-212 and p259-271 in myasthenia gravis (Katz-Levy et al., *Proc. Natl. Acad. Sci. USA* 94: 3200-3205, 1997), the nuclear autoantigen La (SS-B) protein (Bachmann et al., *J. Autoimmun.* 9:747-756, 1996)and the 52-kd Ro(SS-A) protein (Dorner et al., *Hepatology* 24:1404-1407, 1996) in Sjogren's syndrome, and cytochrome P450IID6, the main target antigen of LKM-1 antibody-positive type II autoimmune hepatitis (Lohr et al., *Hepatology* 24:1416-1421, 1996). Other autoimmune antigens will be known to one of ordinary skill in the art.

Other peptide antigens can be identified according to methods used in the foregoing references which describe antigens of tumors, pathogens, allergens and autoimmune disease.

The peptides administered according to the methods described herein can have the amino acid sequence of the naturally processed antigenic peptide (e.g. those listed in Table 1, above). Alternatively, modifications can be made to the amino acid sequence to enhance binding to HLA for presentation to a subject's immune system, while retaining the ability to induce CTLs which recognize the naturally processed peptide. The portions of HLA binding peptides important for binding activity are known (*see, e.g.*, Parker et al., *J. Immunol.* 149:3580-3587, 1992). Variant antigenic peptides which bind more tightly to HLA molecules have been found to be more immunogenic (Parkhurst et al., *J. Immunol.* 157:2539-2548, 1996; Bakker et al., *Int. J. Cancer* 70:302-309, 1997). A general protocol for modification of HLA binding peptides has been suggested by Parker et al. (*J. Immunol.* 152:163-175, 1994; Rammensee et al., *Immunogenetics* 41:178-228, 1995). HLA binding predictions can conveniently be made using an algorithm available via the Internet on the National Institutes of Health World Wide Web site at URL http://bimas.dcrt.nih.gov. Thus one of ordinary skill in the art, with only routine

experimentation, can design synthetic variant peptides which can be administered to induce an immune response in a host. The methods disclosed herein provide a sensitive assay for determining the immunogenic potential of such modified peptides.

The peptides used in accordance with the invention can be prepared by any method known in the art. Typically this entails programming a peptide synthesizer with a desired amino acid sequence, providing the subunits to be incorporated into the extending peptide chain, and purifying the synthesized peptide. All of these steps are well known and practiced routinely in the art. The peptides thus synthesized can incorporate modified amino acids and/or modified inter-amino acid bonds, if desired, to increase stability, reduce proteolysis, or confer some other property to the peptides. Of course, peptides may be made by other methods, including transcription and translation of a nucleic acid which encodes such peptides.

The virus can contain a nucleic acid which encodes one or more antigens, or precursors thereof, including the whole proteins from which the one or more antigens are derived. As used herein, "antigen" embraces a naturally selected antigenic peptide presented by MHC molecules, as well as variants of the naturally selected antigenic peptide which can be prepared according to methods known in the art and disclosed herein. The use of a complete-protein recombinant viral vaccine may be preferable because the host alleles can select the relevant epitope or epitopes for presentation. Host epitope selection recently was demonstrated by Zhai et al (*J. Immunol.* 156: 700-710, 1996), wherein tumor infiltrating lymphocytes recognized five different epitopes presented on the surface of cells transduced with a viral vector expressing a cancer antigen. Alternatively, the use of a viral vaccine which expresses only a portion of a gene which does not encode a functional protein, e.g., a antigen or antigen precursor, may be preferably where it is suspected that the protein may have deleterious effects. Expressing only a portion of the gene permits vaccination against an antigen without concomitant expression of a complete functional gene product in the cells which the vector has infected.

In preferred embodiments, the virus vector for expressing a nucleic acid encoding an antigen is selected from the group consisting of adenoviruses, adeno-associated viruses, poxviruses including vaccinia viruses and attenuated poxviruses, Semliki Forest virus, Venezuelan equine encephalitis virus, retroviruses, Sindbis virus, and Ty virus-like particle. Examples of viruses and virus-like particles which have been used to deliver exogenous nucleic acids include: replication-defective adenoviruses (e.g., Xiang et al., *Virology* 219:220-227, 1996; Eloit et al., *J. Virol* 7:5375-5381, 1997; Chengalvala et al., *Vaccine* 15:335-339, 1997), a

modified retrovirus (Townsend et al., *J. Virol.* 71:3365-3374, 1997), a nonreplicating retrovirus (Irwin et al., *J. Virol.* 68:5036-5044, 1994), a replication defective Semliki Forest virus (Zhao et al., *Proc. Natl. Acad. Sci. USA* 92:3009-3013, 1995), canarypox virus and highly attenuated vaccinia virus derivative (Paoletti, *Proc. Natl. Acad. Sci. USA* 93:11349-11353, 1996), non-replicative vaccinia virus (Moss, *Proc. Natl. Acad. Sci. USA* 93:11341-11348, 1996), replicative vaccinia virus (Moss, *Dev. Biol. Stand.* 82:55-63, 1994), Venzuelan equine encephalitis virus (Davis et al., *J. Virol.* 70:3781-3787, 1996), Sindbis virus (Pugachev et al., *Virology* 212:587-594, 1995),

In particularly preferred embodiments, the virus vector is an adenovirus. An "adenovirus", for the purposes of this invention, refers to an adenovirus that: (1) contains exogenous genetic material that can be transcribed and translated in a mammalian cell and which can induce an immune response in a host, and (2) contains on its surface a ligand that selectively binds to a receptor on the surface of a target cell, such as a mammalian cell, and thereby gains entry to the target cell. The term adenovirus also embraces an adenovirus genome containing exogenous genetic material which encodes a tumor antigen , i.e. an unencapsidated adenovirus. As used herein, "exogenous genetic material" refers to a nucleic acid molecule (e.g., nucleic acid or oligonucleotide), either natural or synthetic, that is not naturally found in an adenovirus. The "exogenous genetic material" is a gene or fragment thereof which encodes an antigen or precursor thereof that can, if necessary, be processed into one or more antigens. The exogenous genetic material can encode more than one antigen, as in a "polytope" nucleic acid.

The genome of an adenovirus is composed of a linear double stranded DNA approximately 36 kilobases in size. The genome comprises, in particular, an inverted repeat sequence (ITR) at each end, an encapsidation sequence (Psi), as well as early genes and late genes. The main early genes are contained in the E1, E2, E3, and E4 regions. Among these early genes, those contained in the E1 region are needed for viral propagation, i.e., replication. The main late genes are contained in the L1 - L5 regions.

The complete nucleotide sequences of adenovirus genomes are known and have been deposited in nucleotide sequence databases. For example, the genome of the adenovirus type 5 has been completely sequenced and is accessible via GenBank accession number M73260. Similarly, portions or even whole genomes of other adenovirus types (type 2, type 7, type 12, and the like) have also been sequenced and deposited in databases.

The nucleic acid encoding a tumor rejection antigen or precursor thereof preferably is

inserted into a region of the adenovirus genome which is not essential to the production of replication-defective recombinant adenoviruses. For example, the nucleic acid preferably is not inserted into regions which contain adenovirus genes encoding proteins which are not easily supplied *in trans*. Thus, the nucleic acid preferably is inserted into the E1 region, which can be complemented (supplied *in trans*) by an adenovirus encapsidation cell line such as 293 cells. Other preferred sites of insertion of the nucleic acid include the E3 region, which is not required for production of replication-defective recombinant adenoviruses, and the E4 region, mutation of which can be complemented by co-transduction with a helper virus or plasmid or by infection of a suitable complementary cell line. Other sites also may be used as will be apparent to one of ordinary skill in the art. In particular, access to the nucleotide sequences of adenovirus genomes enables a person skilled in the art to identify regions of the adenovirus genome suitable for insertion of the nucleic acid encoding a tumor injection antigen precursor.

The replication-defective recombinant adenoviruses of the invention can be prepared by any technique known to the skilled artisan (Levrero et al., *Gene* 101:195 (1991), EP 185 573; Graham, *EMBO J.* 3:2917 (1984)). Generally, adenoviruses are produced by transfection of a recombinant adenovirus genome into an encapsidation cell line. Where several nucleic acids supply different portions of a replication-defective recombinant adenovirus genome, the several nucleic acids can be cotransduced into the encapsidation cell line. In such cases, the process of forming a replication-defective adenovirus genome involves one or more steps of homologous recombination between the different nucleic acids transduced into the encapsidation cell line, in order to generate the recombinant adenovirus genome. Generation of adenoviruses by homologous recombination is exemplified in the examples. After assembly of a complete adenovirus genome (or introduction by transduction a complete adenovirus genome), the adenovirus is assembled by encapsidation of the adenovirus genome with adenovirus coat proteins.

The nucleic acids assembled to prepare a complete replication-defective adenovirus genome can be prepared by any method known in the art. For example, an adenovirus genome can be isolated and then modified *in vitro* by standard methods of molecular biology (see, e.g., *Molecular Cloning: A Laboratory Manual*, J. Sambrook, et al., eds., Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989, or *Current Protocols in Molecular Biology*, F.M. Ausubel, et al., eds., John Wiley & Sons, Inc., New York). The modified adenovirus genome so obtained optionally can be isolated and used to transfect an

encapsidation cell line. Another technique is based on the use of a plasmid carrying a portion of the genome of the recombinant adenovirus, which plasmid is co-transduced with an adenovirus supplying the missing portions of the genome. A complete recombinant adenovirus genome is formed by homologous recombination in the transduced cell line. Other possibilities include the use of prokaryotic plasmids to prepare the recombinant adenovirus genome, followed by transduction of the plasmids into an adenovirus encapsidation cell line.

The adenovirus encapsidation cell line useful for preparation of recombinant adenoviruses is capable of accepting the nucleic acids described above and preferably contains sequences capable of complementing the replication-defective adenovirus genome to permit production of recombinant adenoviruses. Preferably, such sequences capable of complementing the defective portions of the adenovirus genome are integrated into the genome of the cell to avoid recombination with the adenovirus genome. For example, the human embryonic kidney cell line 293 (Graham et al. *J. Gen. Virol.* 36:59, 1977) contains integrated in its genome the left-hand portion of the genome of a type 5 adenovirus. Other cell lines capable of complementing E1 and E4 functions are described in published PCT applications Nos. WO94/26914 and WO95/02697.

Preparation of transduced adenovirus producer cell lines and isolation of adenovirus stock can be performed according to methods standard in the art, as illustrated in the examples below.

Exemplary adenoviruses are replication-defective due to mutation of an adenoviral gene or genes essential for adenoviral replication. As used herein, a "replication-defective" adenovirus is one which is incapable of replicating autonomously in the target cell. Generally, the genome of a replication-defective adenovirus used in the context of the present invention contains mutations or deletions of at least the sequences needed for replication of the adenovirus in the infected cell. Such sequences are well known to those of ordinary skill in the art, and include portions of the E1, E3, and E4 regions of the adenovirus genome. Such regions can be removed in whole or in part, rendered non-functional by mutation, or replaced by other nucleic acid sequences, in particular, a nucleic acid encoding a tumor-rejection antigen precursor. Preferably, the replication-defective adenovirus retains the portions of its genome which are required for encapsidation of the adenovirus genome to form an adenovirus particle. Preferably, the replication-defective adenoviruses of the invention include the inverted repeat sequences (ITRs), a sequence permitting encapsidation, and the nucleic acid encoding a tumor rejection antigen precursor. Replication-defective adenoviruses can contain a modified E1 region which

renders such region non-functional. Preferably, the E1 region is deleted in whole or in part, particularly the portion containing open reading frame 3 (ORF3) and open reading frame 6 (ORF6).

Preferably the adenovirus genome used in the invention is derived from a serotype which does not exert pathogenic effects in humans. Preferred adenovirus serotypes for use in the invention include adenovirus type 2 (Ad2) and adenovirus type 5 (Ad5). Other serotypes useful in this manner will be known to persons of skill in the art. Useful adenoviruses (e.g. non-pathogenic adenoviruses) can be prepared by modification of the genome of a pathogenic adenovirus by art-standard recombinant DNA techniques (see, e.g., *Molecular Cloning: A Laboratory Manual*, J. Sambrook, et al., eds., Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989, or *Current Protocols in Molecular Biology*, F.M. Ausubel, et al., eds., John Wiley & Sons, Inc., New York). Other adenoviruses useful in the invention include those of non-human origin (see, e.g., PCT application WO94/26914)

The adenoviruses described herein are useful for delivering a nucleic acid encoding an antigen or precursor thereof, optionally a whole protein, into a mammalian target cell for *ex vivo* and *in vivo* immunotherapy. Thus the adenoviruses of the invention are useful for delivering to antigen presenting cells nucleic acid molecules that encode antigen precursors, which can be processed by the antigen presenting cells into one or more antigens, e.g., tumor rejection antigens, and presented at the cell surface to enhance the immune system response of the mammalian recipient to the specific antigen.

In some embodiments of the invention, only the antigen peptide is expressed by a recombinant adenovirus. As demonstrated in the Examples below, expression of a tumor antigen peptide by an adenovirus is effective for inducing an immune response against the peptide. Peptides which are larger than the tumor antigen peptides can also be used provided that the larger peptide, when expressed in a host cell, is processed to a peptide which can be presented to the immune system by HLA molecules of the host.

In a preferred embodiment, nucleic acids encoding a series of epitopes, known as "polytopes", are used according to the invention. The epitopes can be arranged in sequential or overlapping fashion (*see, e.g.*, Thomson et al., *Proc. Natl. Acad. Sci. USA* 92:5845-5849, 1995; Gilbert et al., *Nature Biotechnol.* 15:1280-1284, 1997), with or without the natural flanking sequences, and can be separated by unrelated linker sequences if desired. The polytope is processed to generated individual epitopes which are recognized by the immune system for

generation of immune responses.

Thus, for example, for tumor antigens, peptides derived from the MAGE-1 polypeptide, and which are presented by MHC molecules and recognized by CTL or T helper lymphocytes can be combined with peptides from other tumor rejection antigens (e.g. by preparation of hybrid nucleic acids or polypeptides) to form "polytopes". Exemplary tumor associated peptide antigens that can be administered to induce or enhance an immune response are derived from tumor associated genes and encoded proteins including those provided above (e.g., MAGE-1, MAGE-2, MAGE-3, MAGE-4, MAGE-5, MAGE-6, MAGE-7, MAGE-8, MAGE-9, MAGE-10, MAGE-11, GAGE-1, GAGE-2, GAGE-3, GAGE-4, GAGE-5, GAGE-6, BAGE-1, RAGE-1, LB33/MUM-1, DAGE, NAG, MAGE-Xp2, MAGE-Xp3, MAGE-Xp4, tyrosinase, brain glycogen phosphorylase, Melan-A, and MAGE-C1). Preferably the peptide antigens are derived from human polypeptides. Additional peptide antigens and nucleic acids encoding them will be known to one of ordinary skill in the art. One of ordinary skill in the art can prepare polypeptides comprising one or more of the foregoing peptides, or nucleic acids encoding such peptides, according to standard procedures of molecular biology.

Thus polytopes are groups of two or more potentially immunogenic or immune response stimulating peptides which can be joined together in various arrangements (e.g. concatenated, overlapping). The polytope (or nucleic acid encoding the polytope) can be administered in a standard immunization protocol, e.g. to animals, to test the effectiveness of the polytope in stimulating, enhancing and/or provoking an immune response.

The peptides can be joined together directly or via the use of flanking sequences to form polytopes, and the use of polytopes as vaccines is well known in the art (see, e.g., Thomson et al., *Proc. Natl. Acad. Sci USA* 92(13):5845-5849, 1995; Gilbert et al., *Nature Biotechnol.* 15(12):1280-1284, 1997; Thomson et al., *J. Immunol.* 157(2):822-826, 1996; Tam et al., *J. Exp. Med.* 171(1):299-306, 1990). For example, Tam showed that polytopes consisting of both MHC class I and class II binding epitopes successfully generated antibody and protective immunity in a mouse model. Tam also demonstrated that polytopes comprising "strings" of epitopes are processed to yield individual epitopes which are presented by MHC molecules and recognized by CTLs. Thus polytopes containing various numbers and combinations of epitopes can be prepared and tested for recognition by CTLs and for efficacy in increasing an immune response.

It is known that tumors express a set of tumor antigens, of which only certain subsets may be expressed in the tumor of any given patient. Polytopes can be prepared which

correspond to the different combination of epitopes representing the subset of tumor rejection antigens expressed in a particular patient or test subject. Polytopes also can be prepared to reflect a broader spectrum of tumor rejection antigens known to be expressed by a tumor type. Similarly, polytopes can be prepared which include multiple peptides representing a plurality of epitopes of other antigens, e.g., derived from pathogens.

Polytopes can be introduced to a patient in need of such treatment via the use of nucleic acid delivery systems described herein, or as polypeptide structures representing booster doses. One can test the polytope delivery systems in mouse models to determine efficacy of the delivery system. The systems also can be tested in human clinical trials.

Optionally, auxiliary nucleic acids are inserted into the virus genome to enhance or otherwise improve the therapeutic efficacy of the immunotherapy in treating the condition. Exemplary auxiliary nucleic acids for delivery to the mammalian target cell include nucleic acids encoding tumor suppressor genes, nucleic acids encoding antisense mRNA or encoding catalytic RNA that inactivate oncogenes, and nucleic acids that render a target tumor cell more susceptible to an administered drug (e.g., suicide genes encoding, for example, thymidine kinase). Auxiliary nucleic acids also include nucleic acids encoding cytokines that enhance a naturally occurring anti-tumor immunity. Exemplary cytokines which have this function include, e.g., IL-4, TNF, IL-2, IL-12, IL-18 and GM-CSF. Furthermore, nucleic acids encoding costimulatory molecules such as B7-1 and B7-2 can be used (Chamberlain et al., *Cancer Res.* 56:2832-2836, 1996; Townsend and Allison, *Science* 259:368-370, 1993; Chen et al., *J. Exp. Med.* 179:523-532, 1994).

The nucleic acid molecule encoding an antigen or antigen precursor is inserted into the virus genome using conventional recombinant DNA techniques, as exemplified in the Examples. See also, e.g., *Molecular Cloning: A Laboratory Manual*, J. Sambrook, et al., eds., Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989, or *Current Protocols in Molecular Biology*, F.M. Ausubel, et al., eds., John Wiley & Sons, Inc., New York. In the preferred embodiments, a nucleic acid molecule encoding a tumor rejection antigen precursor is inserted into well-defined restriction sites in a portion of the adenovirus genome not required for transcription or translation of the nucleic acid molecule. (*See, e.g.*, the Examples.) For example, the nucleic acid molecule encoding a tumor rejection antigen precursor preferably is inserted in one of the E1, E3 or E4 regions of adenovirus.

In the preferred embodiments, the virus genome further includes a regulatory sequence,

e.g., a promoter region (also referred to as a "promoter"), that is operably coupled to the nucleic acid molecule encoding an antigen or antigen precursor. The regulatory sequence controls the expression of the nucleic acid molecule encoding an antigen or antigen precursor in the target cell. As used herein, a nucleic acid molecule encoding an antigen precursor (the "coding sequence") and regulatory sequences are said to be "operably" joined when they are covalently linked in such a way as to place the transcription or the expression of the coding sequence under the influence or control of the regulatory sequences. If it is desired that the coding sequence be translated into a functional protein, two DNA sequences are said to be operably joined if induction of a promoter in the 5' regulatory sequence results in the transcription of the coding sequence and if the nature of the linkage between the two DNA sequences does not (1) result in the introduction of a frame-shift mutation, (2) interfere with the ability of the promoter region to direct the transcription of the coding sequences, or (3) interfere with the ability of the corresponding RNA transcript to be translated into a protein. Thus, a promoter region would be operably joined to a coding sequence if the promoter region were capable of effecting transcription of that DNA sequence such that the resulting transcript might be translated into the desired protein or polypeptide.

The precise nature of the regulatory sequences needed for gene expression may vary between species or cell types, but shall in general include, as necessary, 3' or 5' non-transcribed non-translated sequences involved with the initiation of transcription and translation respectively, such as a TATA box, CAAT sequence, and the like. Especially, such 5' non-transcribed regulatory sequences will include a promoter region which includes a promoter sequence for transcriptional control of the operably joined gene. Regulatory sequences can also include enhancer sequences or upstream 5' or downstream 3' transcriptional regulatory sequences as desired.

Exemplary promoters that are useful in the invention include constitutive promoters and regulatable promoters (e.g., cell lineage specific promoters, inducible promoters). Exemplary constitutive promoters include promoters derived from cytomegalovirus, a long terminal repeat (LTR) of retroviruses, e.g., Rous sarcoma virus or Moloney murine leukemia virus, and adenovirus E1A promoter, an adenovirus MLP promoter and a SRα promoter. Exemplary tissue or cell specific transcriptional regulatory sequences can be derived from the genes encoding the following proteins: tyrosinase, lipoprotein lipase, albumin, muscle creatine kinase, keratin (K14/K10), globin gene cluster, immunoglobulin heavy chain gene cluster, and involucrin.

Several liver-specific promoters, such as the albumin promoter/enhancer, also have been described (see, e.g., PCT application number PCT/US95/11456, having international publication number WO96/09074, entitled "Use of a Non-mammalian DNA Virus to Express an Exogenous Gene in a Mammalian Cell," hereinafter W096/09074, and the references cited therein). In particular, the alpha-feto protein promoter, can be used to effect expression of a therapeutic polynucleotide(s) in liver tumor cells (but not normal liver cells) for treating liver cancer. Exemplary inducible promoters are described in the following references: *Science* 268:1786 (1995); *TIBS* 18:471 (1993); *PNAS* 91:3180 (1994); *PNAS* 90:1657 (1993); *PNAS* 88:698 (1991); *Nature Biotechnol.* 14:486 (1996); and *PNAS* 93:5185 (1996). An exemplary repressible promoter, the tetracycline repressible system, is described in *PNAS* 89:5547 (1992). Other constitutive, tissue-specific, inducible and repressible promoters will be known by those of skill in the art and thus are not listed here.

The viruses optionally contain one or more sequences that are suitable for use in the identification of cells that have or have not been transduced. "Transduction", as used herein, refers to the introduction of the virus genome into the target cell. Markers to identify cells that have been transduced include, for example, genes encoding proteins that increase or decrease resistance or sensitivity to antibiotics or other compounds, genes which encode enzymes having activities that are detectable by standard assays known in the art and genes which detectably (e.g. visibly) affect the phenotype of the transduced target cells, hosts, or plaques. Exemplary genes that are suitable as markers include a *lacZ* gene, a chloramphenicol acetyltransferase gene, an alkaline phosphatase gene, a luciferase gene, and a green fluorescent protein gene.

The modified viruses of the invention can be delivered to a cell of a subject by methods known to those of ordinary skill in the art. Preferably a subject is injected with the modified virus which includes a nucleic acid encoding an antigen against which modulation of an immune response is desired. However, other methods are also available to the skilled artisan, particularly in cases in which the virus is modified to recognize a certain receptor on the cell of a subject. Methods for delivering whole encapsidated virus include injection, inhalation, ingestion, *in vitro* infection of cells, and the like. For example, when delivering a recombinant adenovirus genome in association with an adenovirus coat (i.e. in the form of an infectious encapsidated adenovirus) to a cell, the cell can be contacted with the adenovirus, and the adenovirus genome can be delivered by receptor-mediated endocytosis via binding of an adenovirus coat protein to a cellular receptor. Methods for delivering non-encapsidated virus include the foregoing methods

and also methods for delivery of nucleic acids to cells familiar to those of skill in the art of molecular biology. For example, when delivering a recombinant adenovirus genome without any associated coat protein, the nucleic acid can be introduced into a cell by transduction using a standard technique such as electroporation, liposome transfection, calcium phosphate precipitation, or a commercially available technology such as the Tfx-50 transfection reagent (Promega Corp., Madison, WI).

If desired, the virus can be modified to target it to certain cell type or tissue. Targeting of a virus to a particular cell or tissue can be accomplished by choice of an appropriate ligand/receptor pair which is specific for the cell or tissue, and incorporation of the ligand into the virus coat. Methods for attaching a ligand to a viral coat are disclosed below. Exemplary receptors (and ligands) include hepatic receptors (hyaluronic acid, collagen, N-terminal propeptide of collagen type III, mannose/N-acetylglucosamine, complement, asialoglycoprotein, tissue plaminogen activator, low density lipoprotein, insulin, ceruloplasmin, enterokinase, carcinoembronic antigen, apamin, galactose/lactose); growth factor/cytokine receptors (hepatocyte growth factor, epidermal growth factor, insulin-like growth factor I, II, interleukin-1α/β, interleukin-2, IL-7, IL-4, γ-interferon, β-interferon, keratinocyte growth factor, TNF-R p55); hormone receptors (prolactin, thyroglobulin, growth hormone, insulin, glucagon, leutinizing hormone, human choriogonadotrophic hormone); nerve cell receptors (neurotensin); antigen presenting cell receptors (immunoglobulin G-Fc receptor); kidney cell receptors (angiotensin II, vasopressin); bone marrow receptors (c-kit, CD-34); keratinocyte and skin fibroblast receptors (very low density lipoprotein, low density lipoprotein, integrins that bind to RGD bearing polypeptides, collagen, laminin); placental receptors (hemopexin, immunoglobulin G-Fc, low density lipoprotein, transferrin, alpha2-macroglobulin, ferritin, insulin, γ-interferon, epidermal growth factor, insulin-like growth factor); muscle cell receptors (insulin, very low density lipoprotein); gut epithelium receptors (cobalamin-intrinsic factor, heat stable enterotoxin of *E. coli*); breast epithelium receptors (heregulin, prolactin); melanocyte receptors (c-kit). Other ligands and receptors will be known to one of ordinary skill in the art.

In addition to the well-known ligand/receptor pairs for delivering a ligand-labeled component to a particular cell type, novel ligands can be identified using phage display procedures such as those described in Hart, et al., *J. Biol. Chem.* 269:12468 (1994). While such filamentous phages could, of course, never be used to deliver genetic material to a cell (because they are single stranded), this methodology is potentially very useful in the discovery of novel

receptor ligand interactions. For example, Hart et al. report a filamentous phage display library for identifying novel peptide ligands for mammalian cell receptors. In general, phage display libraries using, e.g., M13 or fd phage are prepared using conventional procedures such as those described in the foregoing reference. The libraries display inserts containing from 4 to 80 amino acid residues. The inserts optionally represent a completely degenerate or a biased array of peptides. Ligands that bind selectively to a particular type of target cell are obtained by selecting those phages which express on their surface a ligand that binds to the target cell of interest. These phages then are subjected to several cycles of reselection to identify the peptide ligand-expressing phages that have the most useful binding characteristics. Typically, phages that exhibit the best binding characteristics (e.g., highest affinity) are further characterized by nucleic acid analysis to identify the particular amino acid sequences of the peptides expressed on the phage surface and the optimum length of the expressed peptide to achieve optimum binding to the target mammalian cell. Alternatively, such peptide ligands can be selected from combinatorial libraries of peptides containing one or more amino acids. Such libraries can further be synthesized which contain non-peptide synthetic moieties which are less subject to enzymatic degradation compared to their naturally-occurring counterparts. These novel ligands can be attached to the virus surface to deliver the virus to the particular target cell of interest.

At least four procedures are known to those of skill in the art for attaching a ligand to the surface of an virus. These include (1) chemical modification of the virus surface (e.g., galactosylation, cross linking reactions); (2) modification of the virus genome to express a ligand on the virus surface (e.g., a fusion protein formed between the ligand and a functional viral packaging protein); (3) selective binding of a ligand (e.g., a monoclonal antibody, a polyclonal antibody, or functionally active fragments thereof containing an Fc domain) to an virus surface antigen to mediate targeting of the virus to cells that express an Fc receptor on their surface; and (4) modification of the virus genome to form an avidin-labeled virus intermediate to which a biotinylated ligand (e.g., antibody) can be attached.

The viruses of the invention are contacted with the target cell under conditions to permit selective binding of the ligand on the surface of the virus to the receptor on the surface of the target cell and to allow the virus to enter the target cell. Conditions which permit the binding of a receptor to its cognate ligand are the physiological conditions (e.g., the particular pH, ionic strength, viscosity) at which the ligands and receptors are known to bind to one another *in vivo* and the conditions at which the ligands and receptors are known to bind to one another *in vitro*,

such as in receptor assays for determining the presence of a ligand in, for example, a biological fluid. Such conditions are known to those of ordinary skill in the art of receptor-mediated processes, such as receptor-based binding assays and receptor-mediated delivery of therapeutic agents to preselected tissues *in situ*.

In general, the conditions that allow the target cell to live and transcribe the nucleic acid molecule encoding a tumor rejection antigen precursor are the same conditions that permit selective binding of the ligand to the receptor and that allow the virus to enter the target cell. Optionally, the conditions that allow the cell to transcribe the nucleic acid molecule further include the addition of an inducer that activates an inducible promoter to induce transcription and translation of the nucleic acid molecule.

The optimum conditions for inducing the transcription and translation of a nucleic acid molecule encoding a tumor rejection antigen precursor that is under the control of a particular inducible promoter can be determined by one of ordinary skill in the art using no more than routine experimentation. In general, for *in vitro* applications, conventional tissue culture conditions and methods are used to sustain the mammalian cell in culture. For example, the mammalian cell can be allowed to live on a substrate containing collagen, e.g., type I collagen, or a matrix containing laminin, such as described in PCT application number PCT/US95/11456, having international publication number WO96/09074, entitled "Use of a Non-mammalian DNA Virus to Express an Exogenous Gene in a Mammalian Cell," and the references cited therein.

As used herein, "contacting", in reference to the virus and the target cell, refers to bringing the virus into sufficiently close proximity to the target cell to permit the receptor on the target cell to selectively bind to the ligand on the virus. Such conditions are well known to those of ordinary skill in the art. See also, e.g., U.S. patent No. 5,108,921, issued to Low et al. which reports the conditions for receptor-mediated delivery of "exogenous molecules" such as peptides, proteins and nucleic acids to animal cells and U.S. patent No. 5,166,320, issued to Wu et al., which reports the conditions for the receptor mediated delivery of a ligand-gene conjugate to a mammalian cell. For a further discussion of the conditions and mechanisms by which receptor mediated delivery can be used to deliver an exogenous molecule into a target cell, and in particular, into a mammalian cell, see, e.g., S. Michael, et al., *J. Biol. Chem.* 268(10):6866 (1993), "Binding-incompetent Adenovirus Facilitates Molecular Conjugate-mediated Gene Transfer by the Receptor-mediated Endocytosis Pathway"; M. Barry, et al., *Nature Medicine* 2(3):299 (1996), "Toward cell-targeting gene therapy vectors: Selection of cell-binding peptides

from random peptide-presenting phage libraries"; S. I. Michael, *Gene Ther.* 2:660 (1995), "Addition of a short peptide ligand to the adenovirus fiber protein".

The modified virus can be contacted with the targeted mammalian cell *in vitro*, for example, for *ex vivo* immunotherapy or *in vivo* for *in vivo* immunotherapy. As used herein, a "mammalian target cell" refers to a mammalian cell (preferably, a human cell) which contains on its surface a receptor for the ligand that is contained on the surface of the virus (which may be the natural ligands of the virus which allow entry of the virus into cells of its host range). Essentially any mammalian cell can be targeted in accordance with the methods described herein. The cell may be a primary cell or may be a cell of an established cell line. Exemplary cell types that can be targeted are provided above in connection with receptor/ligand pairs. Preferably, the mammalian cell is a cell which contains the immune system components required for the induction of an immune response in the host.

Where the modified virus is contacted with the cell *in vitro*, the target cell subsequently can be introduced into the mammal (e.g., into the portal vein or into the spleen) if desired. Accordingly, expression of the nucleic acid molecule encoding an antigen or antigen precursor is accomplished by allowing the cell to live or propagate *in vitro*, *in vivo*, or *in vitro* and *in vivo*, sequentially. Similarly, where the invention is used to express an antigen or antigen precursor in more than one cell, a combination of *in vitro* and *in vivo* methods are used to introduce the nucleic acid molecule encoding the antigen or antigen precursor into more than one mammalian cell.

In *ex vivo* immunotherapy, as for *ex vivo* gene therapy, the cells are removed from a subject and a nucleic acid (i.e. adenovirus genome) is introduced into (i.e., transduced) the cells *in vitro*. Typically, the transduced cells are expanded in culture before being reimplanted into the mammalian recipient. The procedure for performing *ex vivo* gene therapy is outlined in U.S. Patent 5,399,346 and in exhibits submitted in the file history of that patent, all of which are publicly available documents. In general, *ex vivo* immunotherapy involves the introduction *in vitro* of a nucleic acid which encodes an antigen into a cell(s) of a subject, and returning the genetically engineered cell(s) to the subject to stimulate an immune response. The nucleic acid which encodes an antigen is under the operable control of regulatory elements which permit expression of the nucleic acid which encodes an antigen in the genetically engineered cell(s). In *in vivo* immunotherapy, the target cells are not removed from the patient. Rather, the nucleic acid molecule encoding a tumor rejection antigen precursor is introduced into the cells of the

mammalian recipient *in situ*, i.e., within the recipient. In general, the methods disclosed herein are practiced by using a modified virus, e.g. a replication-defective adenovirus as described herein, in place of the gene therapy vectors disclosed in the prior art in the procedures currently used for administering these vectors (or cells containing these vectors) to the subjects. Such procedures are known to those of skill in the art of human gene therapy.

For *in vivo* immunotherapy, the virus or virus genome is administered to the mammalian recipient, for example, intravascularly, intraluminally (introduction of the adenovirus into body cavities and lumens, such as the genital-urinary tract, gastrointestinal tract, trachea-bronchopulmonary tree or other internal tubular structures), direct injection into a tissue (e.g., muscle, liver), topical application (e.g., eye drops or aerosol application to mucosal surfaces), or intracavitarily (e.g., intraperitoneally or intrathecally (introduction into the cerebrospinal fluid)). Although the ligand/receptor-mediated delivery of the modified virus is the predominant mechanism for targeting delivery of the virus to a particular cell type, delivery to the target cell can further be modulated by regulating the amount of virus administered to the mammalian recipient and/or by controlling the method of delivery. Thus, for example, intravascular administration of the virus to the portal vein or to the hepatic artery can be used to facilitate targeting the virus to a liver cell.

In general, the modified virus can be administered to the mammalian recipient using the same modes of administration that currently are used for adenovirus-mediated gene therapy in humans. Such conditions are adequate for contacting the virus and the target cell under conditions to permit selective binding of a ligand on the surface of the virus to a receptor on the surface of the target cell and to allow the virus to enter the target cell. These conditions are described in the following references: *PNAS* 90:10613 (1993); *Nature Medicine* 1:1148 (1995); *Nature Medicine* 12:266 (1996); *New Engl. J. Med.* 333:832 (1995); and *New Engl. J. Med.* 333:823 (1995). The virus can be administered to the mammalian recipient by intra-vascular injection, intra-organ introduction by, for example, injection into the organ or contacting the virus with the organ in the presence of a tissue permeabilizing agent; and introduction of the virus into body cavities or lumens. Mammalian cells which have been transduced with the virus *ex vivo* can be introduced into the mammalian recipient using the known methods for implanting transduced cells into a human for gene therapy. See, e.g., U.S. Patent No. 5,399,346 ("Gene Therapy") issued to Anderson et al.; PCT International application no. PCT/US92/01890 (Publication No. WO 92/15676, "Somatic Cell Gene Therapy", claiming priority to U.S. Serial

No. 667,169, filed March 8, 1991, inventor I. M. Verma); PCT International application no. PCT/US89/05575 (Publication No. WO 90/06997, "Genetically Engineered Endothelial Cells and Use Thereof", claiming priority to U.S. Serial No. 283,586, filed December 8, 1989, inventors Anderson, W.F. et al.).

The invention is not limited in utility to human immunotherapy, but also provides a method for assessing the effects of immunosuppressive agents in mammalian models such as primates, pigs, sheep, dogs, rodents, and cows. The invention also provides an improved method for testing the effectiveness of antigen peptides as booster agents in a mammal. A virus containing a nucleic acid molecule encoding an antigen can be administered to a group of mammals as a priming immunization. A series of peptides can then be administered to the group of mammals as booster immunizations. The series of peptides can include variants of recognized antigenic peptides and the immune response of each of these variants can thus be assessed to determine the optimal amino acid sequence of immune response boosting peptides. Modifications to the peptides can be made based on known parameters of HLA binding affinity described above, or randomly, and tested for immunogenic potential by the same methodology.

The invention provides other compositions and kits which are useful for practicing the above-described methods. According to a particularly preferred aspect of the invention, kits are provided which contain (a) an adenovirus containing a nucleic acid molecule that encodes a tumor rejection antigen or precursor thereof; and (b) a peptide optionally in adjuvant for administering as booster immunization(s), and optionally, (c) a separate container of adjuvant. Instructions for the use of the adenovirus and peptide(s) can also be included. The components of the kit are sufficient, when administered to a subject, to induce an immune response in the subject against the antigen encoded by the nucleic acid and represented by the peptide. The adenovirus optionally is contained in a pharmaceutically acceptable carrier to form a pharmaceutical composition. The particularly preferred adjuvant, as previously noted, is QS21/MPL.

The pharmaceutical compositions used in the foregoing methods should be sterile and contain a therapeutically effective amount of the modified viruses for priming an immune response (or target cells containing such viruses) in a unit of weight or volume suitable for administration to a patient. As used herein, an effective amount of a virus encoding an antigen means an amount which is sufficient to induce a first immune response. The first immune response can be measured by determining the activity of cytotoxic T lymphocytes. Methods for

measuring cytotoxic T lymphocyte activity include measurement of tumor necrosis factor release by the cytotoxic T lymphocytes and measurement of chromium release as exemplified in the examples. Other assays will be known to one of ordinary skill in the art and can be employed for measuring the level of the first immune response for comparison with the immune response following peptide administration.

Likewise, the peptide compositions for booster immunizations should also be composed of pharmaceutically acceptable components and administered in effective amounts. In general, an effective amount is that amount of a pharmaceutical preparation that alone, or together with further doses, stimulates the desired response. This can be monitored by routine methods or can be monitored according to diagnostic methods of the invention discussed herein.

Where it is desired to stimulate an immune response using a therapeutic composition of the invention, this may involve the stimulation of a humoral antibody response resulting in an increase in antibody titer in serum, a clonal expansion of cytotoxic lymphocytes, or some other desirable immunologic response. The absolute amount will depend upon a variety of factors, including the material selected for administration, whether the administration is in single or multiple doses, and individual patient parameters including age, physical condition, size, weight, and the stage of the disease. These factors are well known to those of ordinary skill in the art and can be addressed with no more than routine experimentation.

More specifically, an effective amount of a peptide antigen means an amount sufficient to modulate the first immune response. Thus, when the peptide antigens are administered in adjuvant, an effective amount is a combined amount of peptide and adjuvant which increases the immune response relative to the first immune response generated by administration of the virus encoding the antigen. When the peptide antigens are administered in a non-adjuvant carrier, an effective amount is an amount which decreases the immune response relative to the first immune response generated by administration of the virus encoding the antigen. The increase or decrease of the immune response can be measured as above for the first immune response; preferably the immune response is measured by the determining the activity of cytotoxic T lymphocytes by measuring tumor necrosis factor release or chromium release from cytotoxic T lymphocytes. An increase in the activity of the cytotoxic T lymphocytes relative to the activity of the cytotoxic T lymphocytes present after the first immune response indicates that the peptide in adjuvant enhanced the first immune response. A decrease in the activity of the cytotoxic T lymphocytes relative to the activity of the cytotoxic T lymphocytes present after the first immune response

indicates that the peptide in a non-adjuvant carrier reduced the first immune response.

The term "pharmaceutically acceptable" means a non-toxic material that does not interfere with the effectiveness of the biological activity of the active ingredients. The term "physiologically acceptable" refers to a non-toxic material that is compatible with a biological system such as a cell, cell culture, tissue, or organism. The characteristics of the carrier will depend on the route of administration. Physiologically and pharmaceutically acceptable carriers include diluents, fillers, salts, buffers, stabilizers, solubilizers, and other materials which are well known in the art.

The doses of antigen-encoding virus and peptide administered to a subject can be chosen in accordance with different parameters, in particular in accordance with the mode of administration used and the state of the subject. Other factors include the particular antigen used for immunization and the desired period of treatment. In general, the viruses of the invention are formulated and administered in the form of doses between 10^4 and 10^{14} plaque forming units (pfu), and preferably 10^6 to 10^{10} pfu. Plaque forming units correspond to the infectious power of an amount of virus. The pfu value of an adenovirus solution, for example, can be determined by infecting a suitable cell culture and measuring, after a time sufficient to allow adenovirus-mediated cell lysis, the number of plaques of infected cells. This and other techniques for determination of the pfu value of an adenovirus stock are known to those of ordinary skill in the art.

In general, booster doses of peptide are formulated and administered in doses between 50 ng and 50 mg, and preferably between 5 μg and 500 μg. Such doses can be administered by injection in one or more sites in the subject. An example of a preferred immunuzation protocol is as follows. A patient can be immunized with peptides for boosting an immune response by injection intradermally (i.d.), subcutaneously (s.c.) or imtramuscularly (i.m.) in 1 to 4 sites of the arms and/or legs of the patient using amounts of peptides ranging from 10 to 300 μg. The patient is immunized 3 to 4 times with an interval of 1 month between immunizations. The volumes for such immunizations usually are 100 to 200 μl for the i.d. route, and 0.5 ml for the s.c. and i.m. routes. Greater amounts of peptide, as indicated above, can be administered. It is also possible to increase the volumes of injections to 2 ml or more with the exception of the i.d. route. Many other protocols will be known to one of ordinary skill in the art, in which the dose amount, schedule of injections, sites of injections, mode of administration (e.g., intra-tumoral) and the like vary from the foregoing. Administration according to the invention to mammals other than

humans, e.g. for testing purposes or veterinary therapeutic purposes, is carried out under substantially the same conditions as described above.

EXAMPLES

Materials and Methods

Mice and immunization

DBA/2J female mice were derived from an inbred colony (Iffa Credo, Lyon, France) and raised in specific pathogen-free conditions in our animal facilities. The mice were between 8 and 12 weeks old. Mice were immunized as indicated with a total amount of 10^7 or 10^8 plaque-forming units (pfu) of recombinant adenovirus injected intradermally (i.d.) into each ear in 50 μl of phosphate buffered saline (PBS), or intraperitoneally (i.p.) with 10^9 pfu in 200 μl of PBS.

Cell lines

Mastocytoma P815 was obtained in a DBA/2 mouse treated with methylcholanthrene. P1.aza^r is a subclone of mastodytoma P815. Antigen-loss variant P1.ist A^-B^-, designated P1.204, is a subclone of mastocytoma P815 obtained from a mouse injected with this P815 cell line. (Uyttenhove et al. *J. Exp. Med.* 157:1040-1052, 1983). The L1210.P1A clone, which expresses the P815A tumor antigen, was obtained after co-electroporation of L1210, a DBA/2 leukemia cell line, with gene *P1A* and pSVtk neoβ. Transfectant clone L1210.P1A.B7.1 was obtained by electroporation of L1210.P1A with a cDNA encoding the murine B7.1 antigen cloned in plasmid pEFBOS (Gajewski et al. *J. Immunol.* 154:5637-5648, 1995). The control L1210.P1A.C clone was obtained by transfecting L1210.P1A with plasmid pEFBOS. BALB/c 3T3 is a fibroblastic cell line and CMS-5 is a carcinoma of BALB/c origin. Dap-L^d is a transfected derivative of DAP-3, a subclone of L fibroblastic H-2^k cell line. These cell lines were cultured in Petri dishes (Falcon 1001, Oxnard, CA) in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % FCS. Cells were incubated at 37°C in an 8 % CO_2 atmosphere.

CTL clone

CTL-P1:5, a clone specific for antigen P815A, was cultivated in 1 ml cultures containing 5 x 10^6 irradiated (30 Gy) DBA/2 feeder spleen cells and 10^5 P1.aza^r cells as stimulators. P1.aza^r cells are killed in medium supplemented with HAT (10^{-4}M hypoxanthine, 3.8 x 10^{-7} M

aminopterin, and 1.6 x 10^{-5} N deoxythymidine). The CTL culture medium was DMEM supplemented with L-arginine (5.5 x 10^{-4} M), L-asparagine (2.4 x 10^{-4} M), L-glutamine (1.5 x 10^{-3} M), glucose (3.5 g/l), HEPES (10^{-2} M), 2-mercaptoethanol (5 x 10^{-5} M) and 10 % FCS (this is referred to as MLTC medium). In addition, cultures contain 50 % of supernatant from secondary mixed lymphocyte culture (MLC) as a source of cytokines. Cultures were maintained in 24 well plates at 37°C in an 8 % CO_2 atmosphere (Uyttenhove et al., 1983).

Mixed lymphocyte-tumor culture (MLTC)

Spleen cells (5 x 10^6) from mice injected two weeks previously with Adeno.P1A or Adeno.βgal were stimulated with 2 x 10^5 irradiated (100 Gy) L1210.P1A.C or L1210.P1A.B7.1 in 2 ml of MLTC medium. The cultures were maintained in 24 well plates at 37°C for 7 days and the responder cells were then tested for cytolytic activity.

CTL stimulation assay

Adenovirus-infected cells were tested for their ability to stimulate the production of TNF by CTL. Briefly, 2000 CTL were added to microwells containing 15,000 target cells in 100 ml in MLTC medium. After 24 hr, 15 ml of the supernatant were collected and incubated with 30,000 cells WEHI-164 clone 13, a TNF-sensitive cell line (Espevik and Nissen-Meyer, *J. Immunol. Methods* 95:99-105, 1986). The cytotoxic effect of the supernatant was determined in a MTT colorimetric assay (Hansen et al., *J. Immunol. Methods* 119:203-210, 1989).

Chromium release assay

The chromium release assay has been described (Boon et al. *J. Exp. Med.* 152:1184-1193, 1980). Briefly, CTL-P1:5 or MLTC responder cells were mixed at various ratios with 2,000 ^{51}Cr-labeled target cells. After 4 hr the radioactivity was measured. BALB/c 3T3, CMS-5 and DAP-L^d targets were incubated with effector cells for 6 to 8 hr.

Construction of recombinant plasmid pAdeno.SRαP1A

Plasmid pcD-SRαP1A was obtained by inserting into the EcoRI site of vector pcD-SRα a 44-bp sequence (SEQ ID NO:1; AATTCGCCGCCATGCTGCCTTATCTAGGGTGGCTGG TCTTCTAG), which codes for the L^d-restricted peptide of murine tumor rejection antigen P815A, preceded by a Kozak consensus start sequence. The SRα promoter system is composed

of the SV40 origin region containing an enhancer and a transcription starting point preceded by a TATA box, followed by an incomplete HTLV-1 LTR and a 16S splicing region. The HindIII-SalI fragment containing the Srα promoter, the *P1A* sequence and the SV40 polyadenylation signal was cloned in the pBluescript vector. It was excised by digestion with SpeI-SalI and inserted into plasmid pAdeno.RSVβgal (Stratford-Perricaudet et al., *J. Clin. Invest.* 90:626-630, 1992) digested by the same enzymes to remove the RSVβgal cassette while retaining the adenoviral sequences (see Warnier et al., 1996 for additional details).

Construction and propagation of recombinant adenovirus Adeno.P1A

The recombinant adenovirus Adeno.P1A was constructed by *in vivo* homologous recombination in cell line 293 (Graham et al., *J. Gen. Virol.* 3659-72, 1977) between pAdeno.SrαP1A and Adeno.dl324 genomic DNA (referred to below as Adeno.def), as described (Strafford-Perricaudet et al., 1992). Briefly, 293 cells were cotransfected with 5 μg of linearized plasmid pAdeno.SRαP1A and 5 μg of the large ClaI fragment of Adeno.def DNA (see Warnier et al., 1996). The recombinant adenovirus was plaque purified and the presence of the transgene was assessed by restriction analysis of the adenoviral DNA. Recombinant adenoviruses were propagated in 293 cells, purified by double cesium-chloride density centrifugation and extensively dialyzed. The viral stocks were stored in aliquots with 10 % glycerol at -80°C and titered by plaque assay using 293 cells.

Example 1: Immunization of DBA/2 mice with Adeno.P1A and boosting with peptide.

76 DBA/2 female mice (8-12 weeks old) were immunized once with a total amount of 10^8 pfu of Adeno.P1A injected intradermally into each ear in 50 μl of PBS. After 3 weeks, 25 mice received at a two-week interval two subcutaneous injections in the two foot pads of a total amount of 50 μg of EE-P1A peptide (SEQ ID NO:2; Glu Glu Leu Pro Tyr Leu Gly Trp Leu Val Phe) mixed with the QS21/MPL adjuvant (SmithKline-Beecham). The final volume was 100 μl (50μl of a PBS solution of the peptide and 50μl of QS21/MPL). The addition of two glutamic acid residues at the N-terminus of the P1A peptide allowed a higher solubility in PBS or adjuvant. Another group of 26 mice was injected in the same way with the peptide in PBS (no adjuvant). A control group of 25 mice received PBS only (no peptide).

Two weeks after the second injection of the peptide, the spleens of the mice were removed and the spleen cells were tested for their capacity to induce an anti-P815A CTL

response as an *in vitro* test of *in vivo* function. 5 x 10^6 splenocytes were restimulated *in vitro* in the presence of either 2 x 10^5 L1210A+B7.1 or L1210A+.C irradiated stimulating cells (100 Gy) in 2 ml of MLTC medium. The cultures were maintained in 24 well plates at 37°C for 7 days. Their lytic activity was then tested in a chromium release assay, as described above, using 2 x 10^3 P511 or P1.204 target cells. P511 is an azaguanine-resistant variant derived from P815 cells expressing P815A antigen. The negative control P1.204 is an antigen-loss variant which does not express the P815A antigen (Uyttenhove et al., 1983; Lethé et al., *Eur. J. Immunol.* 22:2283-2288, 1992). Both target cells were mixed with a 50-fold excess of unlabeled P1.204 cells to prevent non-specific lysis. Each symbol seen in Fig. 1 represents the lytic activity obtained with splenocytes of one mouse. Results are expressed in the number of lytic unit/10^6 cells (L.U.) on a logarithmic scale (Warnier et al., 1996). Mice with lytic activity up to 10 L.U. were considered double positive (++). Mice with lytic activity between 1 and 10 L.U. were scored +, between 0.2 and 1 L.U. were scored ±, and equal to or below 0.2 L.U. were scored -.

Previous experiments (Warnier et al., 1996) demonstrated that immune responses observed after immunization with adenoviruses which express the P815A peptide alone (no booster) were relatively weak and were regularly observed only upon *in vitro* restimulation with irradiated cells which express the costimulatory molecule B7.1. Considering only the L1210.P1A.C stimulating system, 19 mice out of 25 (76 %) displayed a positive response when EE-P1A + QS21/MPL was injected as booster agents, while only 1 mouse out of 25 (4 %) receiving Adeno.P1A and PBS showed a moderate CTL activity. No lytic activity was observed with the 26 mice injected with Adeno.P1A and the EE-P1A peptide in PBS. Adeno.βgal injected mice boosted with the peptide in QS21/MPL were also all negative. This experiment demonstrates the possibility of enhancing the priming response of Adeno.P1A and the importance of adding adjuvant with the peptide.

Example 2: Immunization of DBA/2 mice with Adeno.P1A followed by four booster injections of peptide.

We investigated the possibility of improving the results obtained in Example 1 above by increasing the number of booster injections using peptide, either in the foot pad or *in loco*. It was also of interest to determine if a low dose of Adeno.P1A (10^7 pfu) was sufficient to prime the mice and allow the boost effect of the peptide-adjuvant mixture. We used two doses of Adeno.P1A (10^7 and 10^8 pfu) following the same protocol of injections as described in

Example 1. Each group of infected animals received 50 μg of the EE-P1A peptide mixed in QS21/MPL four times either i.d. in the ears or s.c. in the foot pad. Control groups were infected with Adeno.P1A alone or with Adeno.βgal followed by peptide in QS21/MPL. Mice were bled 15 days after the fourth injection of the peptide and peripheral blood lymphocytes (PBL) were tested for the presence of an anti-P815A CTL response. 3 x 10^5 Ficoll-purified peripheral blood lymphocytes were restimulated *in vitro* in the presence of either 1.5 x 10^5 L1210A+B7.1 or L1210A+.C irradiated stimulating cells (100 Gy) in 0.8μl of MLTC medium. 2 x 10^6 irradiated normal syngeneic feeder spleen cells (30 Gy) were added. The cultures were maintained in 48 well plates at 37°C for 7 days. Their lytic activity was then tested in a chromium release assay using only 1,000 target cells.

The CTL response obtained is represented in Fig. 2. Focusing on the results obtained with L1210.P1A.C stimulating cells, 13 mice out of 14 (93 %) of the primed mice (10^7 or 10^8 pfu) reinjected s.c. displayed variable but positive responses against antigen P815A. One hundred percent of the mice injected i.d. showed a strong cytolytic activity. This is compared with 7 out of 16 (44 %) of control Adeno.P1A primed mice (10^8pfu) and the 4 out of 16 (25 %) control Adeno.P1A primed mice (10^7 pfu).

Example 3: Immunization of DBA/2 mice followed by two or four booster injections.

The same protocol of immunization as described in the Example above was applied in this experiment with the difference at the Adeno.P1A control mice were reinjected with the 50μg of the irrelevant P198 peptide (Sibille et al., *J. Exp. Med.* 172:35-45, 1990) mixed with the QS21/MPL adjuvant. In order to follow the progression of the percentage of positive animals, PBLs were removed and tested for CTL activity after 2 and 4 injections of the peptide.

The results obtained are shown in Figs. 3 and 4. After two injections, 22 out of 30 (73 %) of the 30 mice injected i.d. and 19 out of 30 (63 %) of the mice injected s.c. showed a specific CTL activity. After four injections of the P815A peptide with adjuvant, 28 mice (93 %) injected s.c. and 30 mice (100 %) injected i.d. were positive. Only 14 mice (47 %) from the control group injected with the P198 peptide in QS21/MPL exhibited a cytolytic activity. This is equivalent to the results obtained without any booster injections. This result confirms that the priming with Adeno.P1A followed by subsequent injections of the P1A peptide in QS21/MPL adjuvant allows the obtainment of a strong immunity against antigen P815A in nearly 100 % of the mice. Four booster injections of peptide are preferred, but fewer injections also promote

strong immunity against the antigen.

EQUIVALENTS

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

All references disclosed herein are incorporated by reference.

A Sequence Listing is presented below and is followed by what is claimed.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

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(E) COUNTRY: UNITED STATES OF AMERICA
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(ii) TITLE OF INVENTION: IMPROVED METHODS FOR INDUCING AN IMMUNE RESPONSE

(iii) NUMBER OF SEQUENCES: 36

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(E) COUNTRY: UNITED STATES OF AMERICA
(F) POSTAL CODE: 02210

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: 08/880,979
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(ix) TELECOMMUNICATION INFORMATION:
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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 44 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

AATTCGCCGC CATGCTGCCT TATCTAGGGT GGCTGGTCTT CTAG 44

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Glu Glu Leu Pro Tyr Leu Gly Trp Leu Val Phe
1 5 10

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Glu Ala Asp Pro Thr Gly His Ser Tyr
1 5

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Ser Ala Tyr Gly Glu Pro Arg Lys Leu
1 5

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Glu Val Asp Pro Ile Gly His Leu Tyr
1 5

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Phe Leu Trp Gly Pro Arg Ala Leu Val
1 5

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Met Glu Val Asp Pro Ile Gly His Leu Tyr
1 5 10

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Ala Ala Arg Ala Val Phe Leu Ala Leu
1 5

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: amino acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Tyr Arg Pro Arg Pro Arg Arg Tyr
1 5

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Ser Pro Ser Ser Asn Arg Ile Arg Asn Thr
1 5 10

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Val Leu Pro Asp Val Phe Ile Arg Cys
1 5

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Val Leu Pro Asp Val Phe Ile Arg Cys Val
1 5 10

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

Glu Glu Lys Leu Ile Val Val Leu Phe
1 5

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

Glu Glu Lys Leu Ser Val Val Leu Phe
1 5

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

Ala Cys Asp Pro His Ser Gly His Phe Val
1 5 10

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

Ala Arg Asp Pro His Ser Gly His Phe Val
1 5 10

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

Ser Tyr Leu Asp Ser Gly Ile His Phe
1 5

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

Ser Tyr Leu Asp Ser Gly Ile His Ser
1 5

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

Met Leu Leu Ala Val Leu Tyr Cys Leu
1 5

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

Tyr Met Asn Gly Thr Met Ser Gln Val
1 5

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

Ala Phe Leu Pro Trp His Arg Leu Phe
1 5

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

Ser Glu Ile Trp Arg Asp Ile Asp Phe
1 5

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

Gln Asn Ile Leu Leu Ser Asn Ala Pro Leu Gly Pro Gln Phe Pro
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

Asp Tyr Ser Tyr Leu Gln Asp Ser Asp Pro Asp Ser Phe Gln Asp
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

Glu Ala Ala Gly Ile Gly Ile Leu Thr Val
1 5 10

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

Ala Ala Gly Ile Gly Ile Leu Thr Val
1 5

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

Ile Leu Thr Val Ile Leu Gly Val Leu
1 5

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

Lys Thr Trp Gly Gln Tyr Trp Gln Val
1 5

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

Ile Asp Thr Gln Val Pro Phe Ser Val
1 5

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

Tyr Leu Glu Pro Gly Pro Val Thr Ala
1 5

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

Leu Leu Asp Gly Thr Ala Thr Leu Arg Leu
1 5 10

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

Val Leu Tyr Arg Tyr Gly Ser Phe Ser Val
1 5 10

(2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

Leu Tyr Val Asp Ser Leu Phe Phe Leu
1 5

(2) INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

Lys Ile Ser Gly Gly Pro Arg Ile Ser Tyr Pro Leu
1 5 10

(2) INFORMATION FOR SEQ ID NO:35:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

Tyr Met Asp Gly Thr Met Ser Gln Val
1 5

(2) INFORMATION FOR SEQ ID NO:36:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

Tyr Glu Ile Trp Arg Asp Ile Asp Phe
1 5

CLAIMS

1. A method for inducing an immune response in a mammal against an antigen comprising:

administering to the mammal a virus containing a nucleic acid molecule encoding the antigen or its precursor, in an amount effective to generate a first immune response, and

administering at least one booster dose comprising at least one peptide in an adjuvant, wherein the at least one peptide includes the antigen, in a combined amount effective to enhance the first immune response.

2. The method of claim 1, wherein the antigen is selected from the group consisting of a tumor antigen, and an antigen characteristic of a pathogen.

3. The method of claim 2, wherein the antigen is a tumor antigen and is selected from the group consisting of MAGE-1 (SEQ ID NOs:3 and 4), MAGE-3 (SEQ ID NOs:5-7), BAGE (SEQ ID NO:8), GAGE (SEQ ID NO:9), RAGE (SEQ ID NO:10), GnT-V (SEQ ID NOs:11 and 12), MUM-1 (SEQ ID NO:13), Tyrosinase (SEQ ID NOs:19, 20, 22, 35 and 36), DAGE (SEQ ID NO:33) and MAGE-6 (SEQ ID NO:34).

4. The method of claim 3, wherein the tumor antigen is a MAGE-3 encoded antigen recognized by HLA-A1 (SEQ ID NO:5).

5. The method of claim 1, wherein the nucleic acid molecule encodes a precursor of the antigen.

6. The method of claim 1, wherein the virus is administered by injection.

7. The method of claim 1, wherein the at least one peptide is administered by injection.

8. The method of claim 6 or 7, wherein the injection is intradermal or subcutaneous.

9. The method of claim 1, wherein the adjuvant is selected from the group consisting of saponin adjuvants (e.g. QS21), preferably combined with monophosphoryl lipid A (MPL) or a

derivative thereof, adjuvants based on emulsions, alum, complete and incomplete Freund's adjuvants, and montanide.

10. The method of claim 9, wherein the adjuvant is QS21/MPL.

11. The method of claim 1, wherein at least two booster doses are administered.

12. The method of claim 11, wherein four booster doses are administered.

13. The method of claim 1, wherein the virus is selected from the group consisting of an adenovirus, an adeno-associated virus, a vaccinia virus, an attenuated poxvirus, a Semliki Forest virus, a Venezuelan equine encephalitis virus, a retrovirus, and a Sindbis virus.

14. The method of claim 13, wherein the virus is an adenovirus.

15. A method for reducing an immune response in a mammal against an antigen, comprising:

administering to the mammal a virus containing a nucleic acid molecule encoding the antigen or its precursor, in an amount effective to generate a first immune response, and

administering at least one booster dose comprising at least one peptide in a non-adjuvant carrier, wherein the at least one peptide includes the antigen, in an amount effective to reduce the first immune response.

16. The method of claim 15, wherein the antigen is selected from the group consisting of a allergen, an allograft antigen and an autoimmune antigen.

17. The method of claim 15, wherein the nucleic acid molecule encodes a precursor of the antigen.

18. The method of claim 15, wherein the virus is administered by injection.

19. The method of claim 15, wherein the at least one peptide is administered by injection.

20. The method of claim 18 or 19, wherein the injection is intradermal or subcutaneous.

21. The method of claim 15, wherein at least two booster doses are administered.

22. The method of claim 21, wherein four booster doses are administered.

23. The method of claim 15, wherein the virus is selected from the group consisting of an adenovirus, an adeno-associated virus, a vaccinia virus, an attenuated poxvirus, a Semliki Forest virus, a Venezuelan equine encephalitis virus, a retrovirus, and a Sindbis virus.

24. The method of claim 23, wherein the virus is an adenovirus.

25. A kit for modulating an immune response against an antigen comprising a first container containing an adenovirus which includes a nucleic acid molecule encoding an antigen and a second container containing at least one peptide including the antigen, sufficient to boost an immune response of a mammal to the antigen.

26. The kit of claim 25, wherein the second container further contains an adjuvant.

27. The kit of claim 25, further comprising a third container containing an adjuvant.

28. The kit of claim 25, further comprising instructions for administering the at least one peptide with an adjuvant.

29. The kit of any of claims 26-28, wherein the adjuvant is selected from the group consisting of saponin adjuvants, saponin adjuvants combined with monophosphoryl lipid A (MPL) or a derivative thereof, adjuvants based on emulsions, alum, complete Freund's adjuvant, incomplete Freund's adjuvant, and montanide.

30. The kit of claim 29, wherein the adjuvant is QS21/MPL.

31. The kit of claim 25, wherein the antigen is a tumor antigen selected from the group

consisting of MAGE-1 (SEQ ID NOs:3 and 4), MAGE-3 (SEQ ID NOs:5-7), BAGE (SEQ ID NO:8), GAGE (SEQ ID NO:9), RAGE (SEQ ID NO:10), GnT-V (SEQ ID NOs:11 and 12), MUM-1 (SEQ ID NO:13), Tyrosinase (SEQ ID NOs:19, 20, 22, 35 and 36), DAGE (SEQ ID NO:33) and MAGE-6 (SEQ ID NO:34).

5

32. The kit of claim 31, wherein the tumor antigen is a MAGE-3 encoded antigen recognized by HLA-A1 (SEQ ID NO:5).

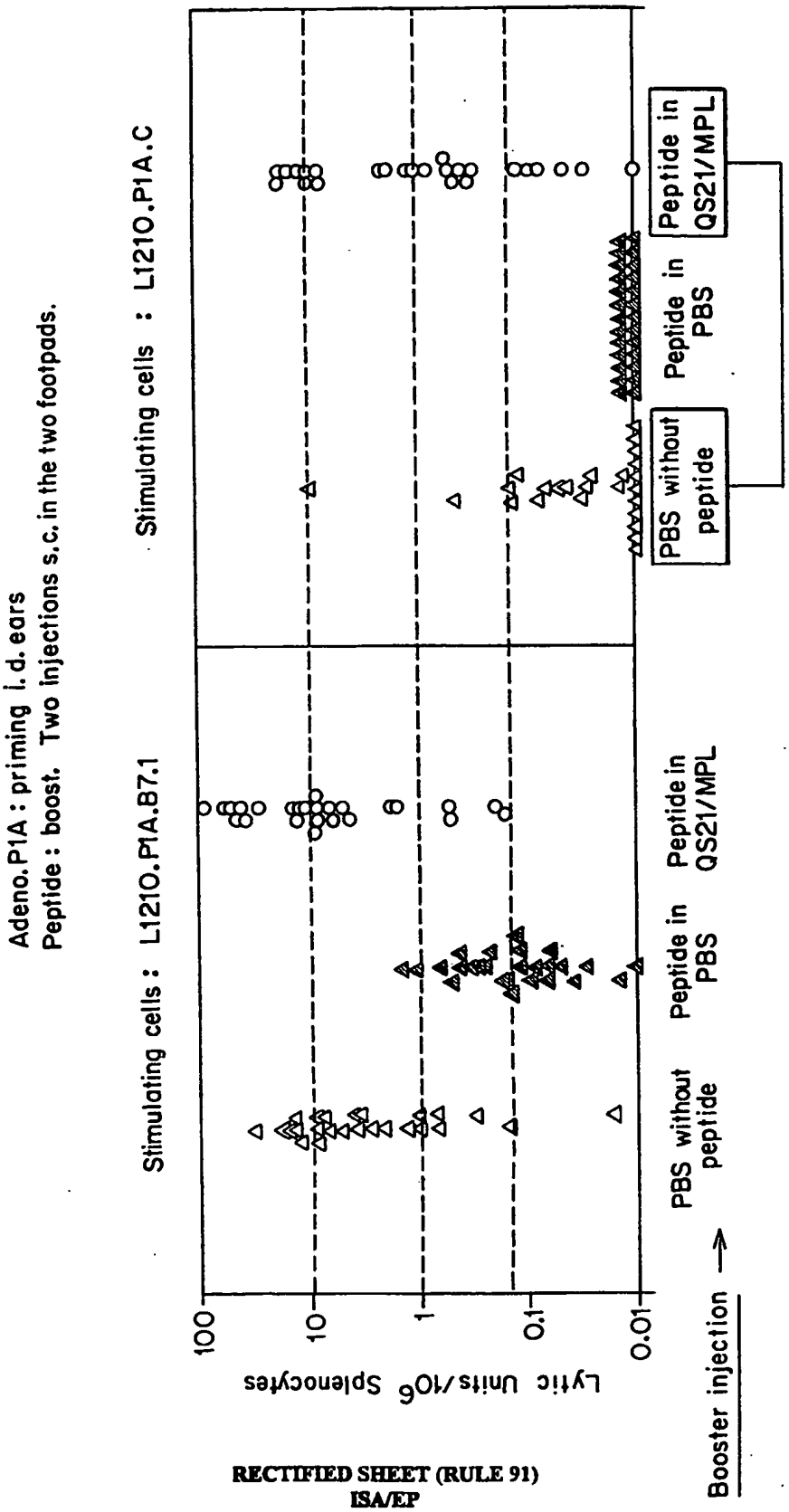
Adeno.P1A : priming i.d. ears
Peptide : boost. Two injections s.c. in the two footpads.
Stimulating cells : L1210.P1A.B7.1
Stimulating cells : L1210.P1A.C
Lytic Units/10^6 Splenocytes
100
10
1
0.1
0.01
PBS without peptide
Peptide in PBS
Peptide in QS21/MPL
Booster injection →

FIG. 1

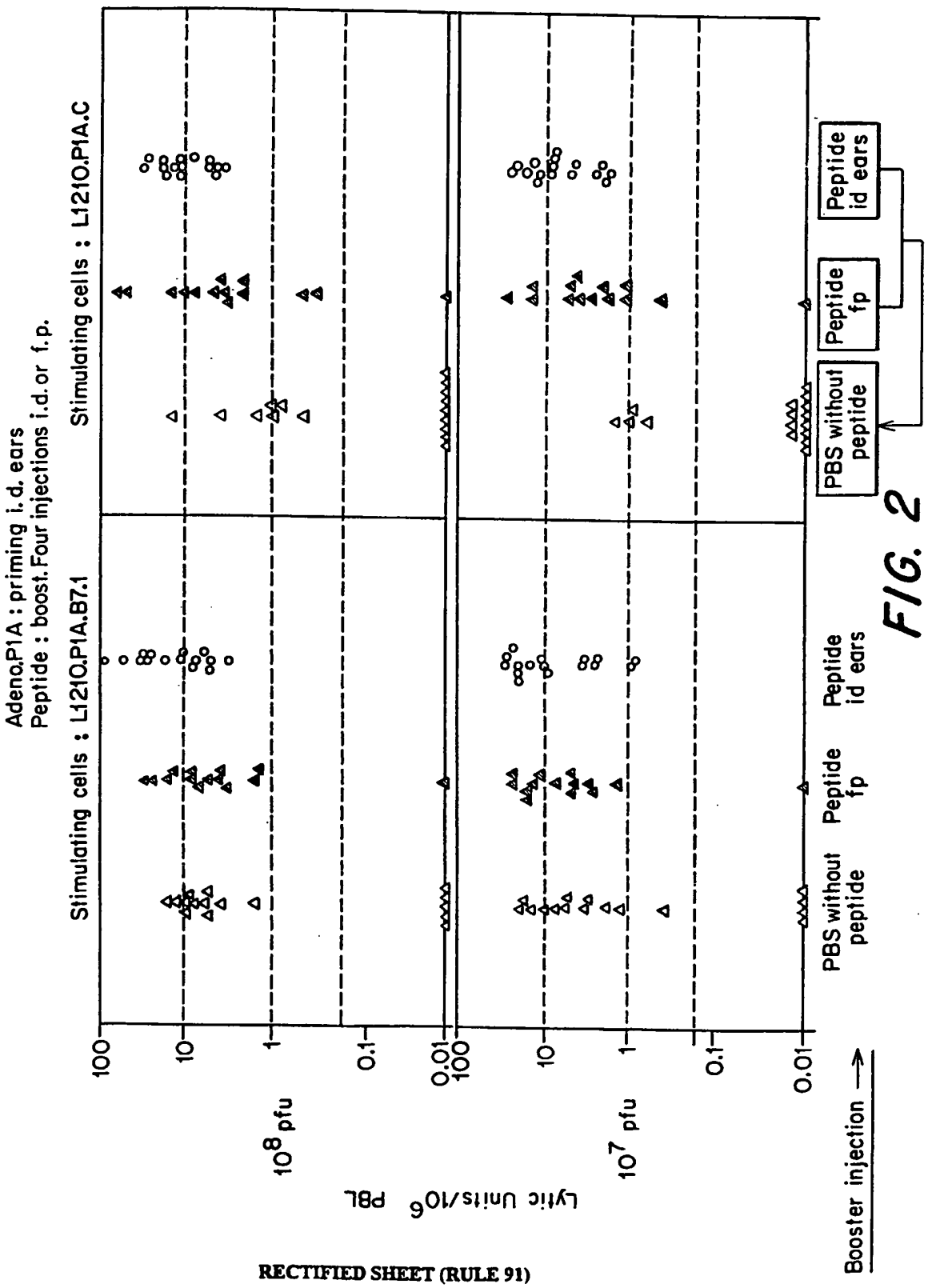
Adeno.P1A : priming i.d. ears
Peptide : boost.Four injections i.d.or f.p.
Stimulating cells : L1210.P1A.B7.1
Stimulating cells : L1210.P1A.C
Lytic Units/10^6 PBL
10^8 pfu
10^7 pfu
100
10
1
0.1
0.01
PBS without peptide
Peptide fp
Peptide id ears
Booster injection

FIG. 2

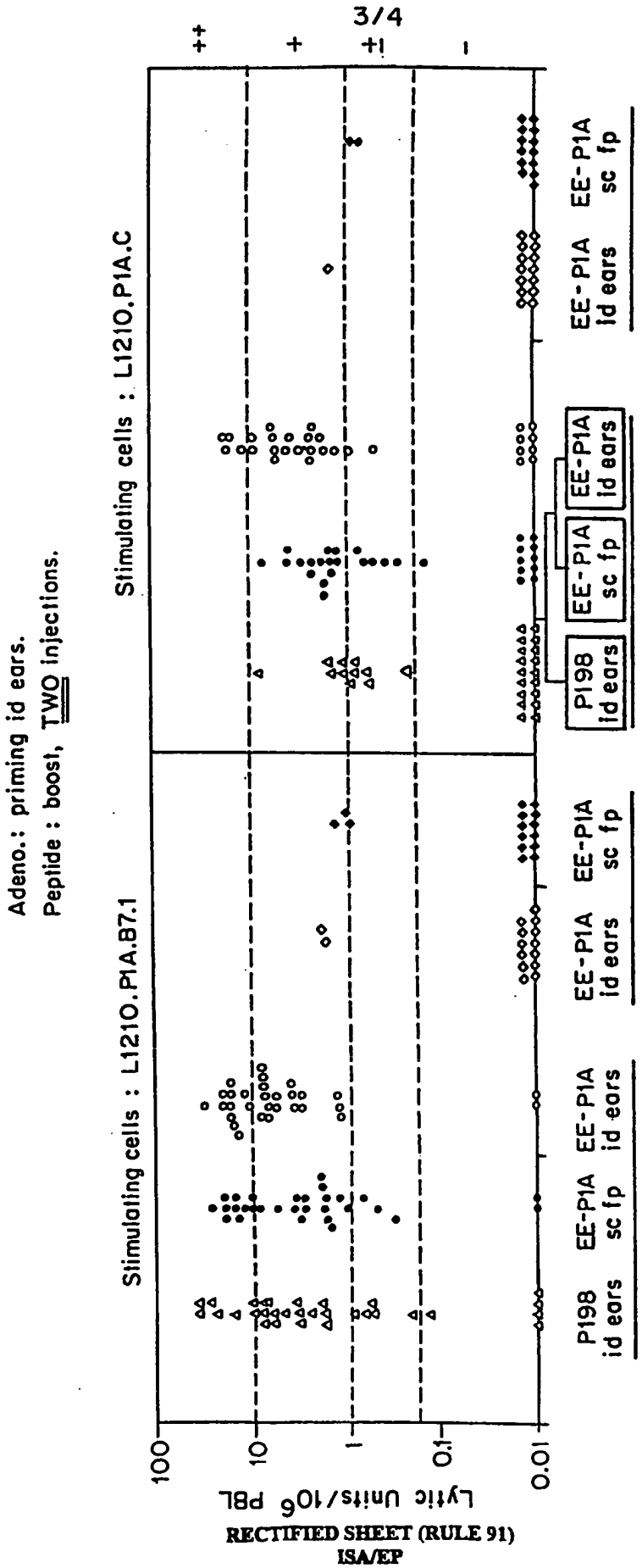
Adeno.: priming id ears.
Peptide : boost, TWO injections.
Stimulating cells : L1210.P1A.B7.1
Stimulating cells : L1210.P1A.C
Lytic Units/10^6 PBL
100
10
1
0.1
0.01
++
+
+!
!
P198 id ears
EE-P1A sc fp
EE-P1A id ears
Adeno.P1A-injected mice
Adeno.βgal-injected mice

FIG. 3

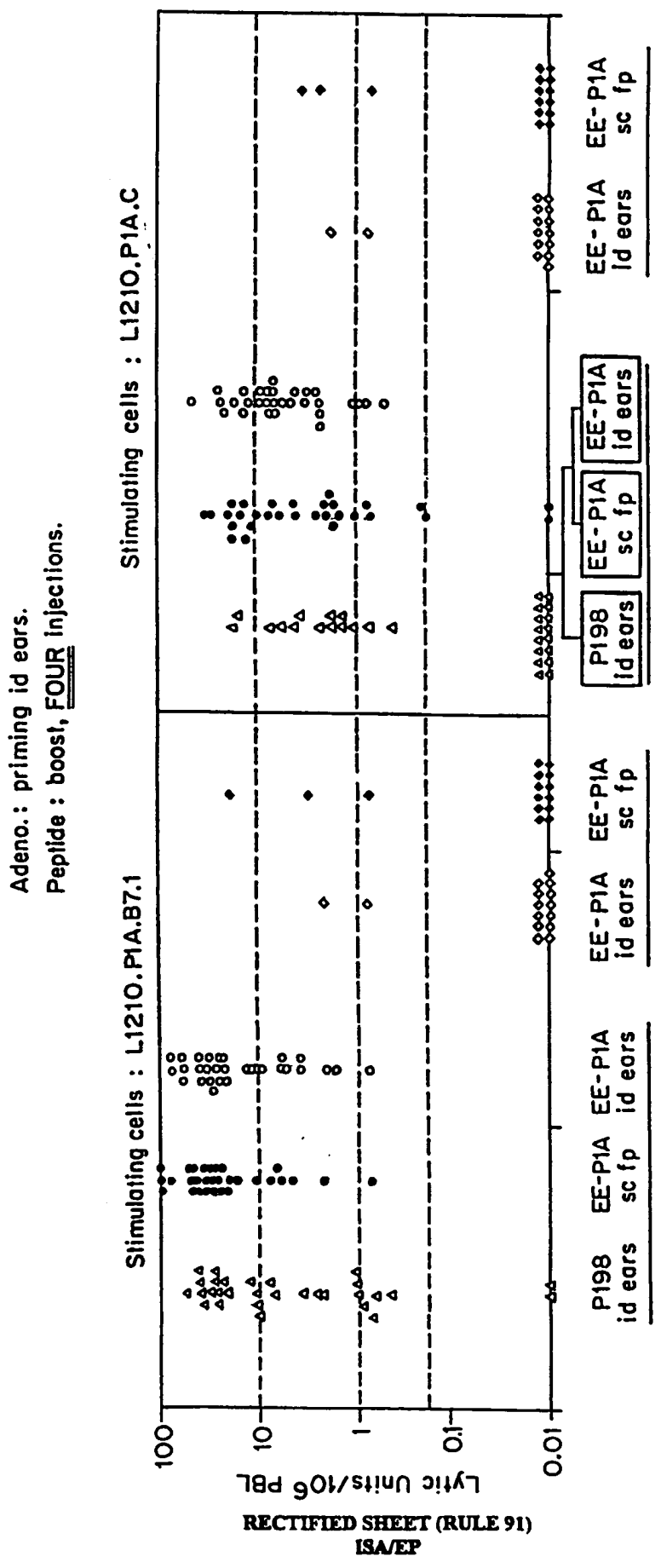
Adeno.: priming id ears.
Peptide : boost, FOUR injections.
Stimulating cells : L1210.P1A.B7.1
Stimulating cells : L1210.P1A.C
Lytic Units/10^6 PBL
100
10
1
0.1
0.01
P198 id ears
EE-P1A sc fp
EE-P1A id ears
EE-P1A sc fp
Adeno.P1A-injected mice
Adeno.Bgal-injected mice

FIG. 4

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